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Site Directed Mutagenesis of Lozenge: A Yeast Two-hybrid Analysis of Transcription Factor Protein Interaction

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Site Directed Mutagenesis of Lozenge: A Yeast Two-hybrid Analysis of Transcription Factor Protein Interaction

A Thesis

**Presented To the Bayer School of Natural and Environmental Sciences
Department of Biological Sciences
Duquesne University**

**In Partial Fulfillment of the Requirements for the
Degree of Master of Science**

By Lailla Boumaza

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**Thesis Committee:
Dr. Jana Patton-Vogt
and
Dr. Sarah Woodley**

May 29, 2007

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I. Forward

Abstract:

Lozenge is a transcription factor. It is a member of the Runx1 homology group that includes Runt, Acute Myeloid Leukemia1, and Core Binding Factor alpha1. Expressed in the fruit fly *Drosophila*, Lozenge contributes to the determination of neuronal and non-neuronal cells by regulating the expression of many fate-determining transcription factors. In addition to Lozenge expression in wide varieties of tissues, it also participates in the pattern formation and specification of the photoreceptors R1/R6 and R7 and cones and pigment cells of the fly eye. A growing number of fate determining transcription factors are influenced by Lozenge during eye development. For example, the directed binding of Lozenge regulates *prospero* expression, which is required for R7 formation. However, for Lozenge to regulate *prospero*, a second transcription factor Pointed, a member of Ets-1 family of transcription factors, is also required. In fact, previous researchers have demonstrated that the DNA binding sites for Lozenge and Pointed are in very close proximity to one another.

During eye development alternative splicing of Lozenge removes an Ets interaction domain, a sequence domain that is contained in the fifth exon. My working hypothesis is that Lozenge binds directly to Pointed before binding to the *prospero* enhancer and that this binding is facilitated by Lozenge Exon 5 alpha helix. Other putative binding sites other than the alpha helix may also be involved in stabilizing the protein-protein interaction. To test this hypothesis I used site directed mutagenesis to alter the amino acid sequence of the putative Lozenge-Pointed interaction domain in the

exon 5 alpha helix of Lozenge. The capacity of the protein interaction is then assayed with yeast two-hybrid analysis. Results of yeast two-hybrid analysis showed that a single proline mutation L439P could weaken the Lozenge-Pointed interaction.

However, the single mutation L439P is not sufficient to completely disrupt the secondary structure of the alpha helix and consequently the Lozenge-Pointed protein-protein interaction.

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II. Introduction & Background

Studying *Drosophila melanogaster* eye development allows one to understand how cells establish fate by cellular contacts and signaling. Cells receive signals from their environment, and are able to respond to particular signaling molecules through signal transduction pathways that change gene expression (Freeman, 2002). Significant cellular diversity arises from a common retinal stem cell giving rise to specific classes of photoreceptor neurons and a range of support cells. In addition, a single class of cell surface receptor proteins, the EGF- receptor which signals through the Ras /MAP Kinase pathway, contributes to the different cellular responses guiding cells to their fate. It is still unknown how a single signaling cascade can specify several different fates, but it is believed that a specific cellular response depends on the developmental state of the cell (Flores et al, 1998. Behan et al, 2003).

The *Drosophila* eye develops from a monolayer epithelium called the eye imaginal disc. This compound eye contains 750-800 ommatidia (Figure 1-A). Each ommatidium has the same number of cells, and each particular cell has a specific role in the perception of light: eight photoreceptors, four cone cells, two primary pigment cells, six secondary, and three tertiary pigment cells, which are shared with neighboring cells (Freeman, 1997). This precise cellular architecture is very important for *Drosophila*'s retina. For many years each ommatidium was considered to be a clone of cells, and their fate was determined by lineage but this was proven to be wrong by Ready and his co-workers 1976. Instead, cells in *Drosophila*'s eye disc derive from a common pool of undifferentiated cells, and they all have the same capability to establish a specific fate

by interacting with other cells (Baker et al. 2001). Until the third larval instars, cells in the eye imaginal disc are still proliferating, without differentiating (Ready et al 1976).

However, around the third instars a groove or a wave known as the morphogenetic furrow (mf) starts sweeping anteriorly across the disc leaving rows of differentiating cells (Figure 1-B). Anterior to the furrow cells are still proliferating, whilst other cells are in G1 arrest as the furrow approaches (Wolff and Ready, 1993). Posterior to the furrow the first cells to establish a fate are the R8 photoreceptor neurons that differentiate under the control of the proneural gene *atonal* (*ato*) (Jarman et al 1994. Baker et al, 1996). Cell R8 in turn recruits photoreceptors pair wise R2 / R5, then R3/R4, which form a precluster of five differentiated cells (Figure 1-C and 1-D) (Ready et al, 1976.

Tomlinson et al, 1985, 1988. Wolff and Ready, 1991 and 1993). This precluster is retained in G1 and the individual cells will never divide again. The G1 arrest is under the control of the *ato* gene and the EGF receptor in R8, and R2, R3, R4, R5 respectively (Baker et al 2001). However, many of the undifferentiated cells that surround the precluster will express Cyclin D and E in order that they may re-enter into the cell cycle in a second mitotic wave (Fig 1B, Ready et al.1976; Wolff and Ready, 1993). After the formation of the eight photoreceptors, the remaining undifferentiated cells are recruited to become cone cells, which induce their neighboring cells to become the primary pigment cells (Cagan and Ready, 1989). Ultimately, the secondary and the tertiary pigment cells are induced to form a mature ommatidium, the extra cells are eliminated by apoptosis (Wolff and Ready, 1991).

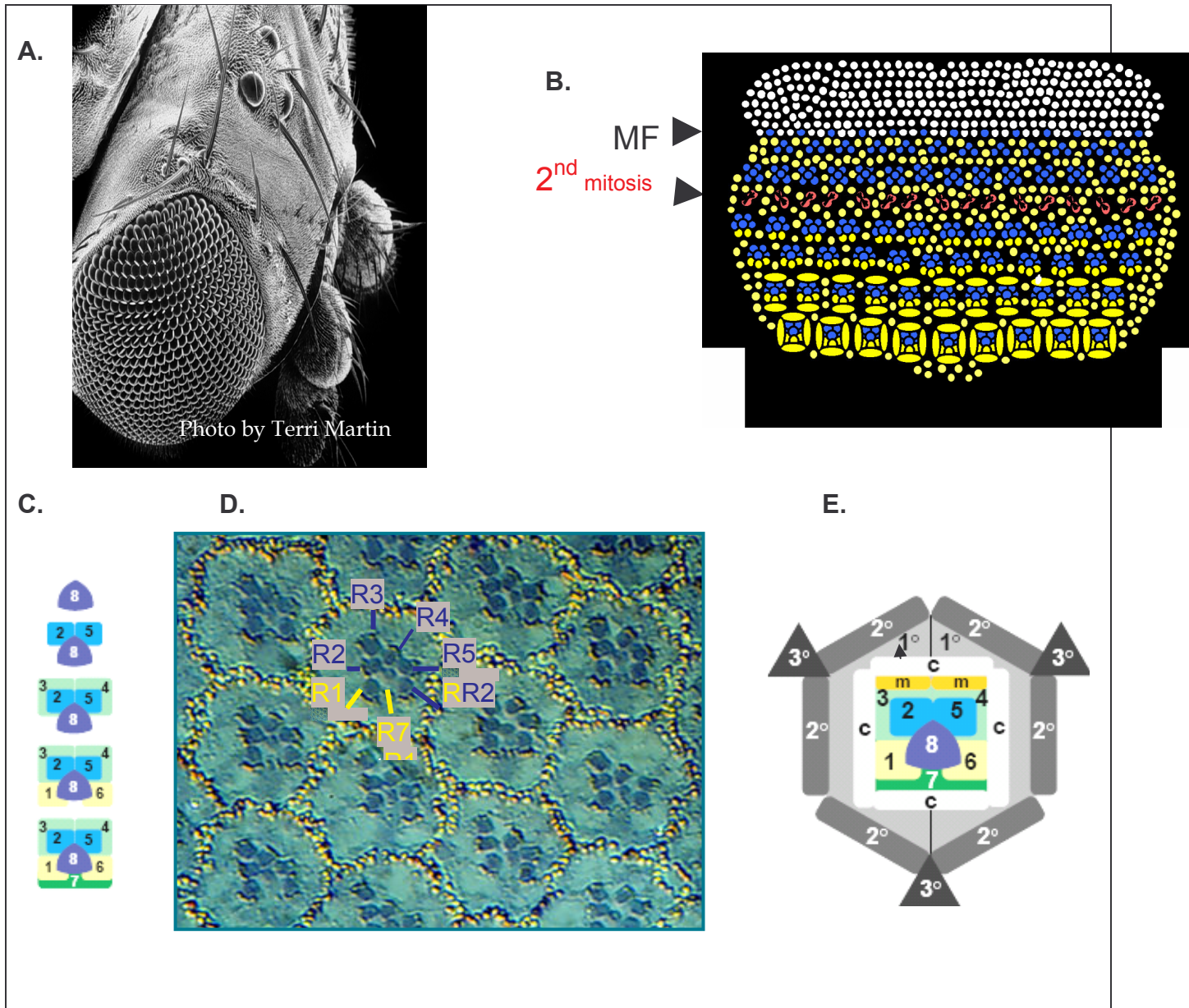
Figure 1: Structure of the adult fly eye

Figure 1: **A:** The wild type compound eye of *Drosophila Melanogaster*. **B:** The morphogenetic furrow in *Drosophila Melanogaster* eye disc the yellow color indicates lozenge expression. **C and D:** Recruited of photoreceptors, R8 differentiates, beginning at top, followed by R2/R5, R3/R4. This is the 5-cell precluster stage. Next follows, R1/R6, and lastly R7 to form the final 8-cell cluster. Taken from (Freeman, 1997).

E. Fully Differentiated Ommatidium. The 8-cell photoreceptor cluster is surrounded by cone cells (c) and two mystery cells (m), and further outward by three types of pigment cells (1°, 2°, 3°). Taken from (Freeman, 1997).

After the so called second wave of cell division, the R1, R6, R7 and cone cell precursors arise from a common pool of undifferentiated cells (Fig 1 B). Together, they share a common development potential and are called “R7 equivalence group.” In other words, they all have the potential to differentiate into an R7 photoreceptor neuron (Hiromi al. 1993). However, only one of these cells actually achieves R7 cell fate. Cone cells are prevented from becoming R7-like cells because they do not come in contact with photoreceptor neuron R8 (reviewed in Dickson 1995).

For each of the photoreceptor neurons, the cell recruitment is initiated through a signal transduction pathway that relies on receptor tyrosine kinases to establish cell fate. These receptors can work individually or in cooperation with other tyrosine kinases. The epidermal growth factor receptor (EGFR) was shown to be one of the most important receptors that induce a signal transduction during eye development. This signal transduction cascade targets phosphorylation of specific transcription factors and permits differentiation (Yang and Baker, 2001). Many ligands are known to bind EGF receptor, but the main ligand involved in EGF receptor activation in the fly eye for cell differentiation is Spitz. Spitz is secreted into the endoplasmic reticulum as an inactive form. However, the protein chaperone Star transports Spitz to the trans Golgi network to be cleaved to its active soluble form by the transmembrane Rhomboid protein. The secreted molecule (spitz) is transported in vesicles to be excreted outside of the cell, where it can induce the activation of the EGF receptor of the neighboring cells (reviewed Freeman, 1997 and 2002). Spitz is initially secreted by the centrally located cells R8 and R2- R5, which diffuse to recruit the neighboring cells R3/R4 and later

R1/R6. However, R7 cells use two different receptors: Sevenless and the EGF receptors, which activate mainly the Ras pathway. Unlike the extracellular paracrine signaling molecule SPITZ, SEVENLESS is activated by a membrane bound juxtacrine ligand called BOSS (Fortini et al, 1992); Boss is presented by the R8 cell to specifically establish R7 fate

In general all of these activated receptors phosphorylate downstream targets including numerous transcription factors that are involved in this developmental process. For example, while R1 and R6 are being induced by the activation of the EGF-R, they are prevented from becoming R7 by the expression of the transcription factor *seven-up*, which is a member of the steroid hormone receptor (Mlodzik et al. 1990). Another transcription factor Prospero is expressed in both R7 and the cone cells (Higashijima et al, 1992), and it has been identified as dependent on the Ras signaling pathway (Kauffman et al. 1996). Both *seven-up* and *prospero* are regulated by Lozenge (Crew et al 1997, Flores et al 1998-2000, Yan et al 1996)

The Transcription Factor Lozenge

The transcription factor LOZENGE is a RUNX1/AML1 homolog (Daga et al 1996). Previous work in Dr. Pollock's laboratory has led to the hypothesis that LOZENGE (LZ) is multifunctional, with separate regulatory programs functioning in the same cell. Previous graduate students have demonstrated that both a full-length 826 amino acid protein (formally known as Lzc3.5) and a shorter 705 amino acid protein lacking exon 5 (formally known as Lz Δ 5) are expressed in the developing eye (Behan et al 2005). In particular, regulation and expression of *lozenge* (*lz*) is central to the development of several cells in the fly eye including photoreceptor neurons R1, R6 & R7, supporting cone cells and pigment cells (Crew et al 1997; Flores et al 1998, 2000; Canon & Banerjee 2000; Nagaraj & Banerjee 2004; Behan et al 2002, 2005). All these cells differentiate from the same pool of multipotent precursor cells. Dr. Pollock has shown that expression of LZ in the R7 equivalence group falls under the receptor tyrosine kinase (EGF-Receptor and Sevenless) regulation of the Ras signaling pathway and the MAP-kinase control of the Ets factors YAN and POINTED (Behan et al 2002). Once LZ is activated, it in turn affects the expression of genes essential for correct differentiation of cells in the eye such as *Bar*, *svp*, *prospero*, *D-Pax-2* (*Spa/shaven*), *runt* and others (Daga et al 1996; Crew et al 1997; Flores et al 2000; Xu et al 2000; Behan et al 2002). Dr. Pollock's group has recently demonstrated that LZ fulfills a second role in eye development, contributing to the specific survival of undifferentiated precursor cells early in development (Siddall et al 2003). Thus, LZ exhibits two distinct roles that we

argue are mediated by the regulated expression to two distinct isoforms of the protein, Lz705 and Lz826 (Fig 2).

The *lozenge* (*lz*) gene has long been recognized as a master regulatory gene involved in the development of the visual system, olfactory system, blood cells and other organs. Beyond *Drosophila* development, this work is of significant interest in that *lz* is a homologue of *AML1* (*acute myloid leukemia1*), the DNA binding alpha subunit of Core Binding Factor. Like LZ, AML1 proteins provide transcriptional regulation for the control of blood cell development (Okuda et al 1996; Wang et al 1996; Lebestky et al 2000; Fossett et al 2003). As mentioned, improper expression of AML1 can lead to the onset of inflammatory diseases or even acute myloid leukemias (Alarcon-Riquelme 2004; Shen & Tsao 2004; Gilliland et al 2004; Yamada & Ymamamoto 2005). Other studies indicate the importance of LZ in olfactory system development of the fly (Gupta et al 1998; Goulding et al 2000; Sen et al 2004) and AML1 in the mammalian olfactory epithelium (TietJan et al 2005). Our recent analysis of YAN regulation of *lz* is reminiscent of the Ets-1 regulation of AML1 (Dittmer 2003). Furthermore, many of the genes that LZ regulates in the fly eye have vertebrate homologues that are also expressed in the developing eye and sensory systems (Cook, 2003). In summary, we have found that not only is LZ structurally similar to AML1/RUNX1, but that both the molecular mechanisms that regulate their expression and the suite of target genes that they regulate are highly conserved between fly and mammals. The continuing characterization of the Lozenge functional cascades is of key significance because these varied tissues and cell types do not all use LZ/AML1 in the same way.

Recently, we demonstrated that in Lz826, exon 5 facilitates protein-protein interaction with the Ets-1 factor POINTED (PNT2) (Behan et al 2005). Shalini Singh performed site directed mutagenesis of LZ protein to demonstrate the importance of amino acid charge structure a putative alpha helix protein motif in the core of the exon 5 protein domain.

The central hypothesis that I will test is that Lz826 exon 5 alpha helical integrity is important for LZ-PNTP2 interaction by using site directed mutagenesis to introduce proline into the alpha helix (Fig 3) (Fig 2 C).

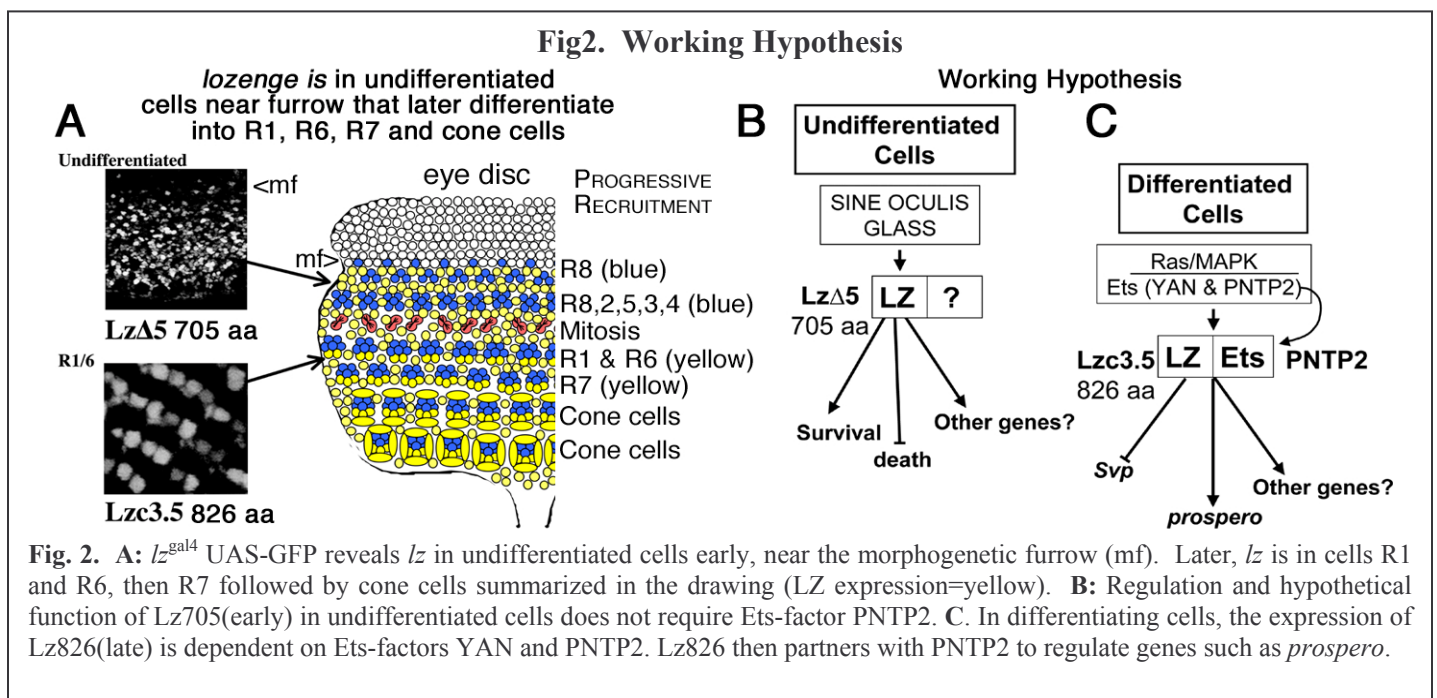


Figure 3:

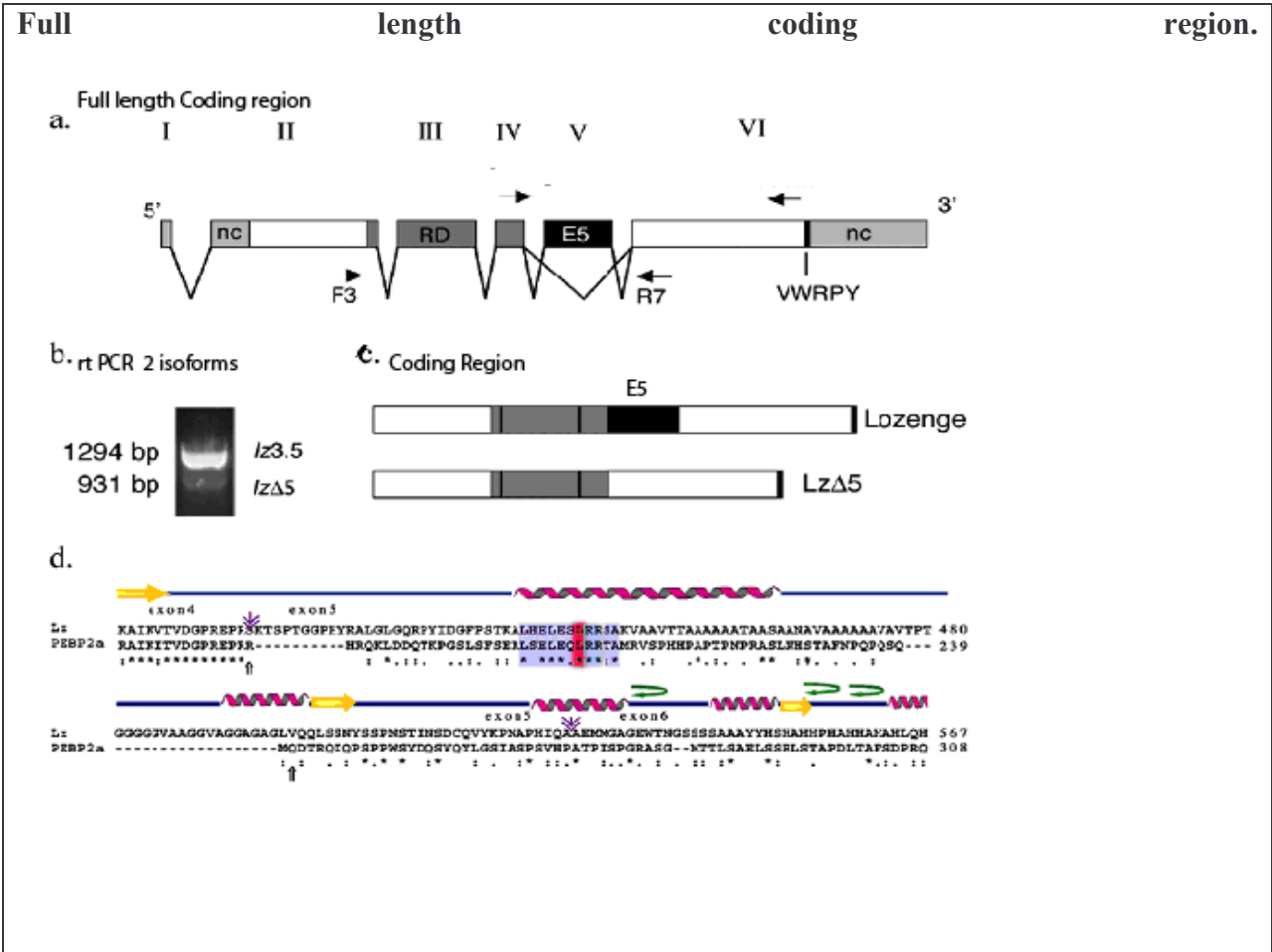
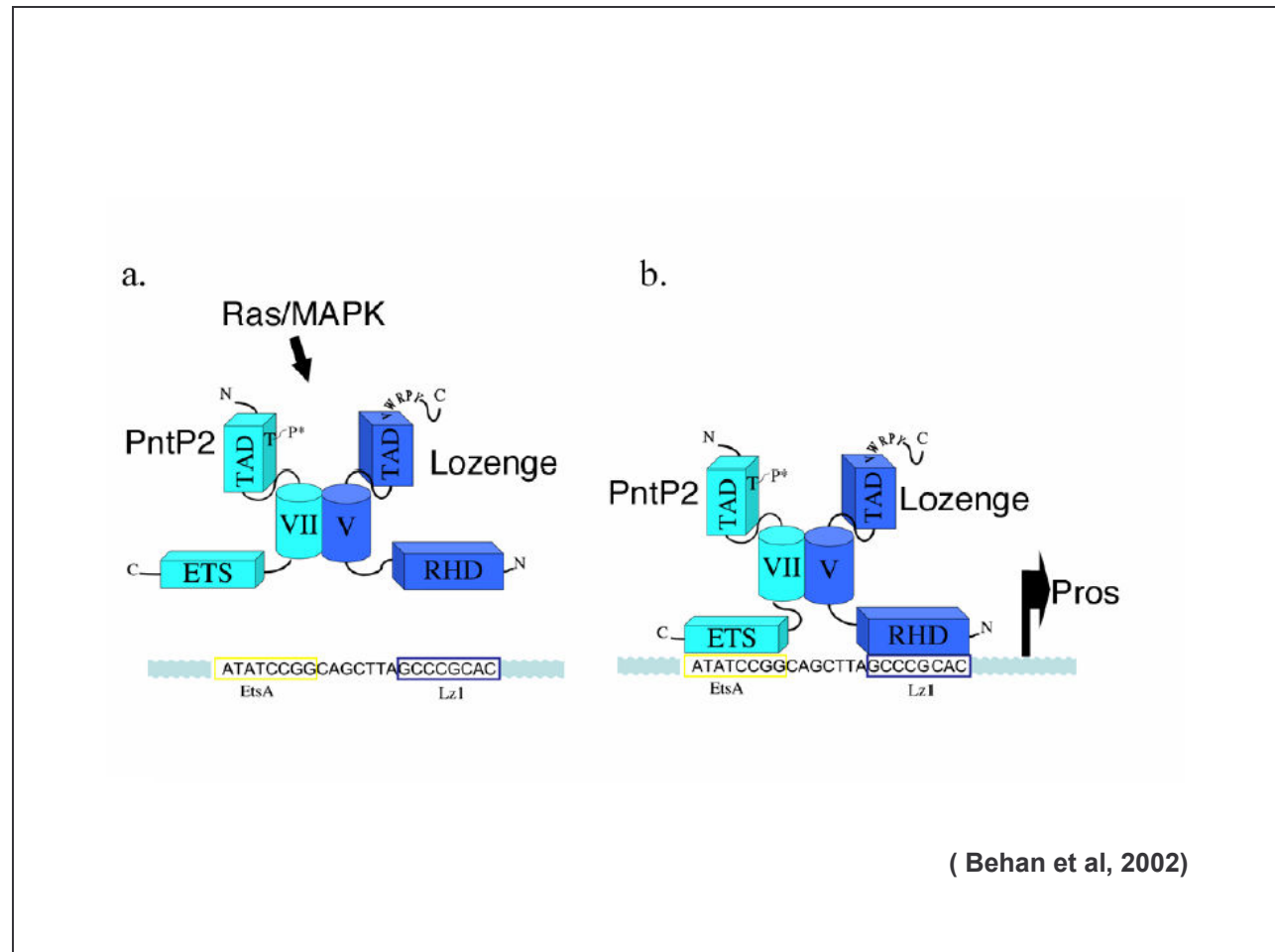


Figure 3: **A:** Shows lozenge full-length transcript (lzc3.5). **B:** RT-PCR on mRNA of tissue from the eye imaginal disc reveals two isoforms of lozenge in gel electrophoresis (lzc3.5) and (lzcΔ5). **C:** Coding regions of both lozenge isoforms **D:** Alignment of Lozenge and murine RUNX1 protein isoform PEBP2αB1, the alpha helix in Lozenge Exon V has a blue box with a high degree of sequence conservation. The red box highlights the conserved leucine that is disrupted by site directed mutagenesis of a proline and analyzed using Yeast two-hybrid analysis.

Figure 4: Model shows the physical interaction of Lonzenge and Pointed proteins before binding *prospero* DNA and down regulating its expression



Specific aims:

1. Generate mutants of both *Lozenge* full length (*Lz c3.5*) and *Lozenge* exon V peptide (*LzE5*) to alter the secondary structure of the α helix by:
 - Using site directed mutagenesis to substitute the amino acid leucine with proline (L439P).
2. Generate Yeast two hybrid expression clones with the Gal4 activation domain and the Gal4 binding domains.
3. Test for physical interaction between Pointed (PntP2) with *Lozenge* full length (*Lzc3.5*), mutant *Lozenge* full length (*Lzc3.5* SDM), *Lozenge* Exon V peptide (*LzE5*), and mutant *Lozenge* Exon V (*LzE5* SDM) using yeast two hybrid system as developed by (Fields and Song 1989).
4. Measure the strength of the putative protein-protein interaction using a standard quantitative assay.

III. Material and methods:

I. Site Directed Mutagenesis:

In vitro site directed mutagenesis is a very efficient method that allows studying protein structure and functioning in molecular biology and protein engineering. The Stratagene Quick Change XL site directed mutagenesis kit (catalog # 200516) permits mutation in any double stranded plasmid and generates mutants with greater than 80% efficiency.

Full-length lozenge and lozenge exonV in pCR-blunt templates were used to generate mutants that change Leucine (L439 P) to proline, which should produce a kink in the alpha helix of the exon V. Perturbation of the secondary structure of exonV, which is involved in LZ/PNTP2 protein-protein interaction may contribute to a conformational change of Lozenge protein that could affect protein-protein interaction between LZ and PNTP2.

1. Primer design:

1-1. Forward and reverse primers were designed individually according to the desired proline mutation and following the manufacturer's instructions.

Both Primers have the desired mutation and must anneal to the same sequence on opposite strands of the plasmid.

Mutation of interest is in the middle and flanked by 15 bases of correct sequence on both sides.

Length of the primers is 33 base pairs

Primers were PAGE purified

The primers were synthesized and purified by Integrated DNA

Technologies (www.idtdna.com) .

In the previous work two contiguous amino acids Arginine (R440 and R441) that are located in the conserved site of LZ exon V alpha helix sequence were

mutated to glutamic acid (R440E and R441E; Behan et al 2005). The secondary structure of the alpha helix was kept intact but only the charges were reversed.

In the current study a kink is introduced in this alpha helical structure by changing leucine to proline.

Forward primer= SDMF

Tm=75.4 C, length= 33 bp

5'CTT GGC GGA CCG CCG GGG CGA CTC CAG CTC GTG 3'

Reverse primer= SDMR

Tm75.4 C, length= 33 bp

5'CAC GAG CTG GAG TCG CCC CGG CGG TCC GCC AAG 3'

*

DGFPSTKALHELES	PRRS	AKVAAV	Lz	SDM	(L439P)
DGFPSTKALHELES	SLRR	AKVAAV	Lz	WT	425-451
GSLSF	SERLSE	QLRRTAMRVSP	AML1		190-214
*					

1-2. Primers dilution:

Primers were diluted to 100ng final concentration in DI H₂O.

2. Mutant strands synthesis (Thermal Cycling):**2-1. Control reaction preparation:** a 50 µl control reaction was set-up

following the manufacturer instructions in a 0.5 ml tube on ice:

5 µl of 10X reaction buffer
2 µl (10ng) pWitescript 4.5-Kb control plasmid (5ng/µl)
1.25 µl (125ng) of oligonucleotide control primer #1 (34-mer (100ng/ µl)
1.25 µl (125ng) of oligonucleotide control primer #2 (34-mer (100ng/µl)
1µl of dNTP mix
3 µl of QuickSolution
36.5µl of nanopure water (ddH₂O) to a final volume of 50µl
Then add
1µl of pfu Turbo DNA polymerase (2.5U/µl).

2-2. Sample reaction preparation: Double stranded DNA

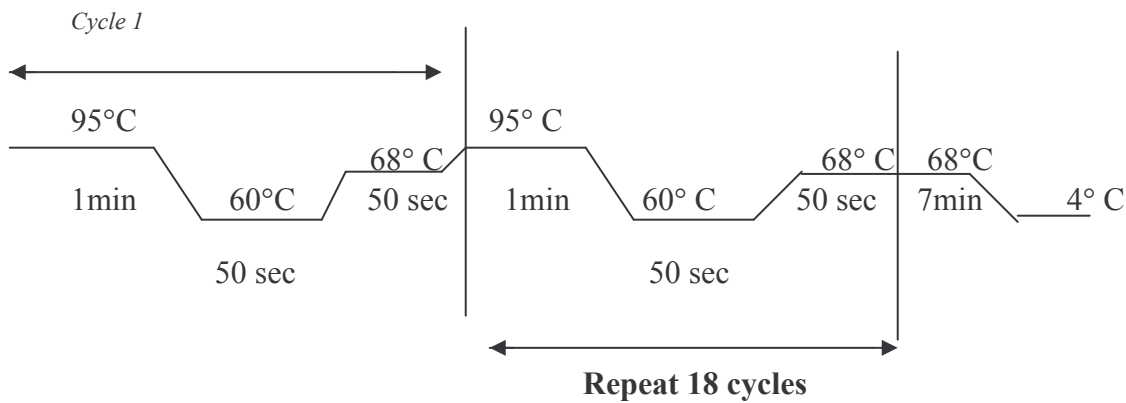
Lozenge full length (Lz c3.5) and separately lozenge exonV

(LzE5) both in pCR-blunt were used as templates. A 50µl reaction

was assembled in in 0.2 ml tube on ice as follows:

5 µl of 10X reaction buffer
1µl (50ng) of pCR blunt-Lz/ pCR blunt exonV
1.25 µl (125ng) of SDMF primer
1.25 µl (125ng) of SDMR primer
1 µl of dNTP mix
3 µl QuickSolution
37.5 µl of nanopure water to a final volume of 50 µl.
Then add
1 µl of pfu Turbo DNA polymerase (2.5 U/ µl)

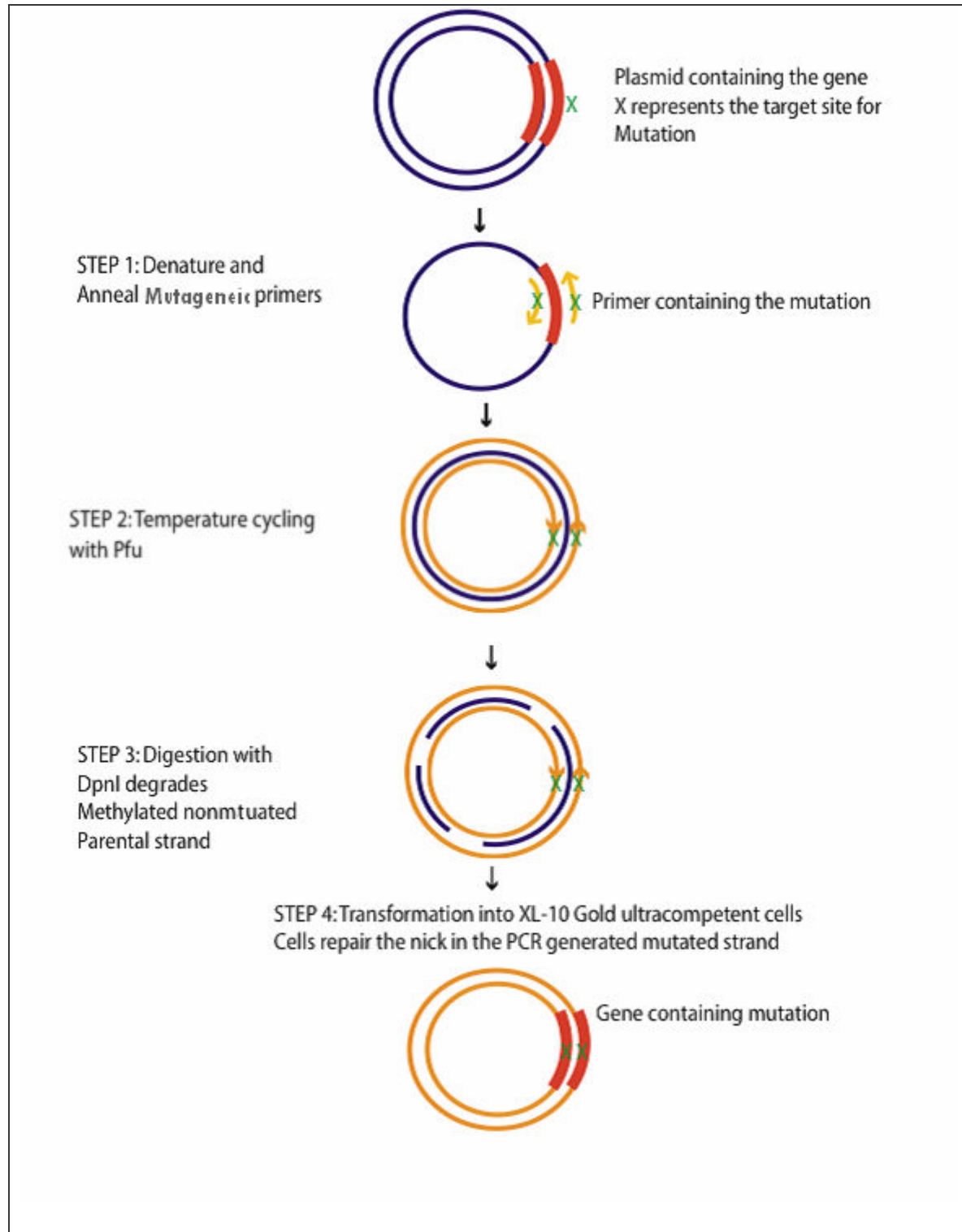
The following cycling parameters were used:



After the thermocycling process, the tubes were placed on ice for 2 minutes to cool the reaction to $<$ or equal to 37°C. 10 μ l of the amplified product was checked by electrophoresis in 1% agarose gel.

2.3. Dpn I digestion of the amplified products:

1 μ l of the Dpn I restriction enzyme (10 U/ μ l) was added directly to each amplification reaction. Each reaction mixture was mixed thoroughly by pipetting up and down several times and spun down in a microcentrifuge tube for 1 minute. The reactions were incubated at 37°C to digest the parental supercoiled ds DNA (Figure 5).

Figure 5: Overview of the Quick Change XL site directed mutagenesis method.

3. Transformation of XL10-Gold Ultracompetent Cells:

The XL10-Gold ultracompetent cells provided with the kit were gently thawed on ice, and 45 μ l of Ultracompetent cells were aliquoted in the prechilled 14 ml BD Falcon polypropylene round-bottom tubes. 2 μ l of the β -mercaptoethanol mix provided with the kit was added, and the tube contents were swirled gently. The cells were incubated for 10 minutes, swirling gently was done every 2 minutes. 2 μ l of the Dpn I- treated DNA from each control and sample reaction was transferred to the tubes with the cells. In addition, a control reaction was performed to check transformation efficiency by using 1 μ l 0.01 ng/ μ l of pUC18. The transformation reactions were swirled gently to mix and incubated on ice for 30 minutes. The tubes were heat-pulsed in a 42 °C in a water bath for 30 seconds, and incubated on ice for 2 minutes. A 0.5 ml of preheated (42 °C) NZY+ broth was added to each tube. The tubes were incubated at 37 °C for 1 hour with shaking at 225-250 rpm. A 250 μ l aliquot of each transformation reaction was plated on LB- Kanamycin agar plates for Lz pCR blunt and Exon V pCR blunt containing 80 μ g/ml X-gal and 20 mM IPTG. 5 μ l of pUC18 transformation control was plated in a pool of 200 μ l of NZY+ broth, and all transformed plates were incubated overnight at 37°C.

Table 1: Volume of cells plated

Reaction type	Volume to plate
pWhitescript mutagenesis control	250 μ l
pUC18 transformation control	5 μ l (in 200 of NZY+ broth)
Sample mutagenesis	250 μ l on each of two plates

II. DNA isolation via miniprep:

10 ml of LB broth that contains kanamycin was inoculated with a white single colony from the transformation plates and incubated overnight at 37° C with shaking at 250 rpm. Cells were harvested and DNA was isolated using Wizard plus SV Miniprep DNA purification system by promega (catalog #A1340). The DNA was analyzed by gel electrophoresis in 0.8% agarose gels to confirm the products' sizes of lozenge insert- plasmid PCR blunt.

III. DNA sequencing:

Primers were designed to sequence the mutant plasmid inserts to confirm the site directed mutagenesis *lz Exon 5 (LzExon5 SDM)* and *lz c3.5 (lzc3.5SDM)*. The following website was used to design the listed below primers:

<http://Frodo.wimit.edu/cgi-bin/primer3/primer3> www.cgi

Table 2: primers used to sequence *lozenge* mutants.

<i>lzc3.5SDM</i>	5' CCA AAG CCA TCA AGG TGA C3'	T _m = 54° C
<i>lzcExon5SDM</i>	5' ACT ACC GAG CTC TGG GTC 3'	T _m =56°C

Spectrophotometric measurement of the absorbance at 260 nm was used to calculate the DNA concentration for each DNA sample and 300-400ng of DNA of each mutant clone was sent for sequencing to the University of Pittsburgh School of Medicine, Biomedical Research Support Facilities, DNA Sequencing Core Facility (<http://www.pitt.edu/~rsup/>).

IV- 80° C glycerol stock of mutant clones:

After the sequences were checked for the desired mutation, a –80 °C glycerol stock was prepared for each mutant clone. This was done using screw-capped plastic freezing vials. To each vial a 0.6 ml overnight culture was added to 0.4 ml of 20% glycerol, the tube contents were mixed and each tube was properly labeled and stored at –80°C freezer.

V. Subcloning:

1. Construction of fusion gene:

Restriction sites were integrated in to each clone to facilitate in frame ligation to the yeast expression clones. PCR primers with specific terminal restriction sites were designed to amplify each of the following candidates clones: *lozenge*, *pointed Pnt* P2, *lozenge* Exon 5 and their corresponding mutants. Each PCR product listed above was cloned into both yeast plasmid containing the GAL4 binding domain (pGBKT7-BD domain) and the plasmid containing the GAL4 activation domain (pGADT7-AD domain).

1- 1. Primers design:

Primers similar to those designed by Shalini Singh (Singh 2004) were used to introduce compatible restriction enzymes sites (Table 3 and 4). Each of Singh's (2004) primers had 2 additional bases added at the 5'ends to allow for more efficient digestion by the restriction enzymes.

Table 3: List of primers used for DNA amplification and the expected PCR product sizes along with the expected size when ligated to yeast plasmids.

Fusion genes	Primer sequences	Melting Temperature/length	PCR product sizes (kb)	Yeast plasmids+	Yeast Plasmid +insert (kb)
Lozenge (Lz)	GBTK7LzF=GCGAATTCATGCATTGTCATCTCCTGGCGGA GBTK7LzR=GCCCCGGGTCAATAGGGTCGCCACACCT	Tm69.9° C Length32 Tm72.2 C Length 28	2.8 kb	pGBKT7 (7.3 kb)	10.1kb
	GADT7LzF=GCGAATTCATGCATTGTCATCTCCTGGCGGA GADT7LzR=GCCCCGGGTCAATAGGGTCGCCACACCT	Tm69.9° C Length32 Tm72.2 C Length 28		pGADT7 (8 kb)	10.8kb
Lozenge mutant (LzSDM)	GBTK7LzF=GCGAATTCATGCATTGTCATCTCCTGGCGGA GBTK7LzR=GCCCCGGGTCAATAGGGTCGCCACACCT	Tm 69.9° C Length32 Tm72.2 C Length 28	2.8 kb	pGBKT7	10.1kb
	GADT7LzF=GCGAATTCATGCATTGTCATCTCCTGGCGGA GADT7LzR=GCCCCGGGTCAATAGGGTCGCCACACCT	Tm69.9° C Length32 Tm72.2° C Length 28		pGADT7	10.8kb
Lozenge Exon5 (LzE5)	GBTK7LzE5F=GCGGATCCGTCCGACCGGTGGTCC GADT7LzE5R=GCCTGCAGTGCTTGTATGTGCGGCGC	Tm 70.5 C Length 24 Tm 70.1 C Length 26	0.4kb (400bp)	pGBKT7	7.7kb
	GADT7LzE5F=GCCCCGGGTAGTCCGACCGGTGGTCCG GADT7LzE5=GCGGATCCTGCTTGTATGTGCGGCGTT	Tm 74.4 C Length 27 Tm 70.2 C Length 29		pGADT7	8.4 kb
+ Lozenge Exon5 SDM (LzE5SDM)	GBTK7LzE5F=GCGGATCCGTCCGACCGGTGGTCC GADT7LzE5R=GCCTGCAGTGCTTGTATGTGCGGCGC	Tm 70.5 C Length 24 Tm 70.1 C Length 26	0.4kb (400bp)	pGBKT7	7.7 kb
	GADT7LzE5F=GCCCCGGGTAGTCCGACCGGTGGTCCG GADT7LzE5=GCGGATCCTGCTTGTATGTGCGGCGTT	Tm 74.4 C Length 27 Tm 70.2 C Length 29		pGADT7	8.4kb
Pointed (Pnt 2)	GBTK7PF=GCGGATCCGTATGGAATTGGCGATTGTAA GBTK7PR=GCCTGCAGCTAATCCACATCTTTTTCTCAAC	Tm 64.6 C Length 30 Tm 63.5 C Length 33	2.5 kb	pGBKT7	9.8 kb
	GADT7PF=GCCCCGGGTATGGAATTGGCGATTGTAA GADT7PR=GCGGATCCCTAATCCACATCTTTTTCTCA	Tm 66.5 C Length 30ntd Tm 62.6 C Length 31ntd	2.5kb	pGADT7	10.5 kb

Table 4: Represents the different restriction sites for each sample clone.

<i>Yeast plasmids</i>	Lozenge DNA in pCR-blunt vector	Restriction enzymes	Primer sequences
pGBTK7 (7.3kb) Binding Domain Plasmid	<i>Lzc3.5 pCR-blunt</i> <i>Lzc3.5 SDM pCR-blunt</i>	XmaI / EcoRI	<i>GBTK7LzF</i> =GCGAATTCATGCATTTCATCTCCTGGCGGA <i>GBTK7LzR</i> =GCCCCGGGTCAATAGGGTCGCCACACCT <i>GBTK7LzE5F</i> =GCGGATCCGTCCGACCGGTGGTCC
	<i>LzExon5 pCR-blunt (IzE5)</i> <i>LzExon5SDM pCR-blunt (IzE5)</i>	PstI / BamHI	<i>GADT7LzE5R</i> =GCCTGCAGTGCTTGTATGTGCGGCGC <i>GBTK7PF</i> =GCGGATCCGTATGGAATTGGCGATTGTAA <i>GBTK7PR</i> =GCCTGCAGCTAATCCACATCTTTTTTCTCAAC
	<i>Pointed (PntP2)</i>		
pGADT7 (8kb) Activation Domain Plasmid	<i>Lzc3.5 pCR-blunt</i> <i>Lzc3.5 SDM pCR-blunt</i>	XmaI / EcoRI	<i>GADT7LzF</i> =GCGAATTCATGCATTTCATCTCCTGGCGGA <i>GADT7LzR</i> =GCCCCGGGTCAATAGGGTCGCCACACCT <i>GADT7LzE5F</i> =GCCCCGGGTAGTCCGACCGGTGGTCCG
	<i>LzExon5 pCR-blunt (IzE5)</i> <i>LzExon5SDM pCR-blunt (IzE5)</i>	XmaI / BamHI	<i>GADT7LzE5</i> =GCGGATCCTGCTTGTATGTGCGGCGTT <i>GADT7PF</i> =GCCCCGGGTATGGAATTGGCGATTGTAAA <i>GADT7PR</i> =GCGGATCCCTAATCCACATCTTTTTTCTCA
	<i>Pointed (PntP2)</i>		

1-2. Amplification:

PCR products of above listed DNA candidates were generated using stratagene PCR kit (cat # 200516). A total reaction volume of 50 μ l for each sample was set up in a sterile 0.2 ml PCR tubes on ice as follows:

10 X buffer	5 μ l
Quick solution	3 μ l
Forward primer	1.25 μ l (100ng/ μ l)
Reverse primer	1.25 μ l (100ng/ μ l)
dNTP	1 μ l
Plasmid DNA	3- 4 (300-400 ng/ μ l)
Nanopure H2O	up to 50 μ l
Pfu Turbo	
DNA polymerase	1 μ l (2.5 U/ μ l)

Every reaction was gently mixed and then briefly centrifuged. All tubes were immediately placed in an eppendorf thermocycler. The program was set-up at 95° C denaturation for 1 minute to start, then 50 seconds per cycle. The annealing temperature was at 60° C for 50 seconds, which was followed by an extension period at 68° C for 1 minute/kb of the gene or gene fragment. This cycle was repeated 18 cycles and finally terminated with an extension time at 68° C for 7 minutes for all samples (Table: parameters). All PCR products were analyzed by gel electrophoresis on 0.8% agarose gels.

1-3. Gel clean-up:

PCR products were purified from the agarose gel to remove PCR leftover products. Samples were run on 0.8 % agarose gel at 80 volts for 75 minutes to permit the isolation of the DNA fragments. Ethidium bromide labeled gels, the DNA bands of interest were illuminated with long wavelength UV light and excised with a clean razor blade. The Qiagen clean-up kit (cat# 28704) was used to purify DNA following the manufacturer instructions. DNA concentration was quantified by spectrophotometric measurement as described above.

1-4. Ligation to pSC-B vector:

In order to digest successfully *Iz c3.5* and *pointed* P2, PCR products of both genes were ligated to pSC-B vector using strataClone blunt PCR Cloning kit (catalog # 240208). A control insert reaction was performed as well. The ligation reaction mixture was prepared by combining in order the following components:

- 3 μ l StrataClone Blunt Cloning Buffer
- 4 μ l of PCR product (200ng)
- 1 μ l StrataClone Blunt Vector Mix

The reaction was gently mixed by pipetting, and incubated at room temperature for 5 minutes. When the incubation was complete, the reaction was placed on ice for transformation into cells.

1-4.1 Transformation of the ligation reaction and DNA purification:

StrataClone SoloPack competent cells were thawed on ice for each ligation reaction and also for both control insert and control transformation. 2 μ l of each cloning reaction mixture was added to the transformation reaction. The transformation reaction was incubated on ice for 20 minutes, heat-shocked at 42°C for 45 seconds, and then incubated again for on ice for 2 minutes. 250 μ l of pre-warmed SOC medium was added to the transformation reaction, and the competent cells were allowed to recover for 1 hour at 37°C with mild agitation. 100 μ l of the samples transformation mixture, 25 μ l of the control insert, and 30 μ l of pUC18 control transformation were plated on LB-X-gal plated with the appropriate antibiotic and incubated overnight at 37°C.

Cells were harvested from 5 ml overnight culture and DNA was purified using StrataPrep Plasmid Miniprep kit (cat # 400761) following the manufacturer instructions. The DNA concentration was estimated by spectrophotometric measurements as described above.

1-5. Restriction digestions:

1-5.1. Digestion by Xma1 and EcoR I:

Each of the clones for *lozenge* full length, and *lozenge* full length SDM and the corresponding yeast expression vectors pGBKT7 and pGADT7 were digested with Xma1 and EcoR1 restriction enzymes (Table 5). The reaction mixture was done on ice, and a sequential double digestion was done due to buffer incompatibility of the two enzymes.

Digestion by Xma1 (New England Biolabs cat # R0 180S) was done first followed by a DNA clean-up using agarose gel electrophoresis and QiAquick Gel extraction Kit (Cat # 28704). The cleaned DNA was subjected to Eco RI (New England Biolabs cat # R0101S) digestion, and DNA was again purified. The buffer was kept to 1/10th and BSA to 1/100th of the total reaction volume. Each enzymatic reaction was set up in order as follows:

Table 5: Restriction digests Xma1 / EcoRI for subcloning

1 st Xma1 digestion Xma1	Amount (μl)	2 nd EcoR I digestion	Amount (μl)
Nanopure H2O to make up 35 μl total volume	12.15	H2O to make up to 35 μl	8.6-10.6
NE buffer 4	3.5	NE EcoR I buffer	3.5 μl
BSA	0.35	BSA	0.35 μl
DNA (Samples PCR products) ~ 0.9 μg	18	Digested Xma1 DNA from gel clean up (~1μg).	18-20 μl
Gently mix the reaction by pipetting			
Xma1 (10,000 U/ml)	1	EcoRI (20,000 U/ml)	0.5 μl
Mix by pipetting, incubate at 37° C for 1hour 30minutes and terminate the reaction at 65° C for 20 minutes in a water bath			

1-5.2 Digestion by XmaI and BamHI:

Each of the clones for *Pointed P2*, *Lozenge Exon V*, mutant *Lozenge Exon V*, and pGADT7 yeast plasmid were first digested by XmaI. Agarose gel DNA purification of the XmaI digest was followed by a second BamHI digestion (Promega cat #R6021). A control reaction was also performed as well, and the reactions digest for both enzymes were set up as follows:

Table 6: Restriction digests XmaI / BamHI *Pointed P2*, *Lozenge Exon V*, mutant *Lozenge Exon V*, and pGADT7 yeast plasmid

1 st XmaI digestion	Amount (μl)	2 nd BamHI digestion	Amount (μl)
Nanopure H ₂ O to make up 35 μl total volume	10.55	the purified Xma digest	19
NE buffer4	3.5	10X Buffer E	3.5
BSA	0.35	BSA (10 μg/ μl)	0.35
DNA (~ 0.8-0.9 μg)	20	Nanopure H ₂ O to make up 35 μl of total volume	

Mix the reactions gently by pipetting,

of XmaI	0.6	BamHI (10U/μl)	0.6
The reaction digest for XmaI was incubated at 37°C for 90 minutes.		The reaction digest for BamHI was incubated at 37° C for 120 minutes	

All reactions were terminated at 65°C for 20 minutes or by adding 6X loading dye.

1-5.3. Digestion by PstI/ BamHI.

Each of the clones for *Pointed (P2)*, *LozengeExonV(Lz E5)*, mutant *Lozenge ExonV (LzE5 SDM)*, and the pGBTK7 yeast plasmid were first digested by PstI Promega (cat #R6111). The purified DNA was subjected to BamHI digestion. The same restriction enzyme set up was followed as indicated by the above Table 6.

2- Yeast plasmids dephosphorylation and ligation:

Both restriction digested yeast plasmids pGADT7 and pGBKT7 were dephosphorylated by using calf intestinal alkaline phosphatase (CIAP) Fisher Scientific (cat # BP3217-1). CIAP catalyzes the hydrolysis of 5'-phosphate groups. This enzyme was used to prevent recircularization and religation of linearized cloning vehicle DNA by removing phosphate groups from both 5'-termini. The alkaline phosphatase was diluted for immediate use in 1X reaction buffer to a final concentration of 0.01U/ μ l. Each pmol of DNA ends will require 0.01U CIAP. The following formula was used to calculate the number of ends:

$$1\mu\text{l of 1,000 bp DNA} = 1.52\text{pmol DNA} = 3.03\text{pmol of ends (cat \# BP3217-1)}.$$

The reaction was assembled as follows:

DNA (up to 10 pmol of 5'-ends)	40 μ l
CIAP 10X Reaction buffer	5 μ l
Diluted CIAP (0.01u/ μ l)	0.5 μ l

The reaction was incubated at 37° C for 30 minutes. Another aliquot of 0.5µl diluted CIAP was added with an additional 30 minutes of incubation. The reaction was terminated by addition 6x loading dye to the samples, which are going to be purified by gel electrophoresis and the Qiagen kit as described above.

Ligation samples for both yeast plasmids listed above were prepared using T4 DNA ligase promega (cat # M1801), and a 1:3 ratio of vector-to-insert was used. The conversion of molar ratios to mass ratios for plasmid and insert DNA fragment was calculated using the following formulae:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The 10 µl reaction was set-up as follows:

Vector	X µl (200ng)
Insert	X µl (600ng)
Ligase	1 µl
10X buffer	2 µl
T4 Ligase	0.5 µl
H ₂ O	2.5 µl

The ligation reaction was incubated overnight at 4° C.

3- Transformation in H101 cells.

The ligation reaction was transformed in H101 competent cells that were provided by Dr. Castric's lab. The cells were thaw on ice, a 100 µl of competent cells were aliquoted in the prechilled 14 ml BD Falcon. 4 µl from each ligation reaction was added to the cells, and swirled gently. The reaction was incubated on ice for 30 minutes, then heat-shocked for 30 seconds in a 42°C

water bath. The tubes were immediately returned on ice for additional 2 minutes incubation, and 500 μ l of preheated NZYE broth was added. The tubes were incubated at 37° C with shaking for one hour. A 150 μ l of cells were plated in 50 μ g/ml Kanamycin and 100 μ g/ml ampicillin LB agar plates. The plated were incubated at 37° C for overnight.

4- DNA purification via miniprep and quatification:

5 ml of LB broth that contains the appropriate antibiotic was inoculated and incubated at 37°C with shaking. DNA purification was done using StrataPrep Plasmid Miniprep kit (cat # 400761). Each sample concentration was quantified by spectrophotometric measurement.

5- Sequencing and –80° stock:

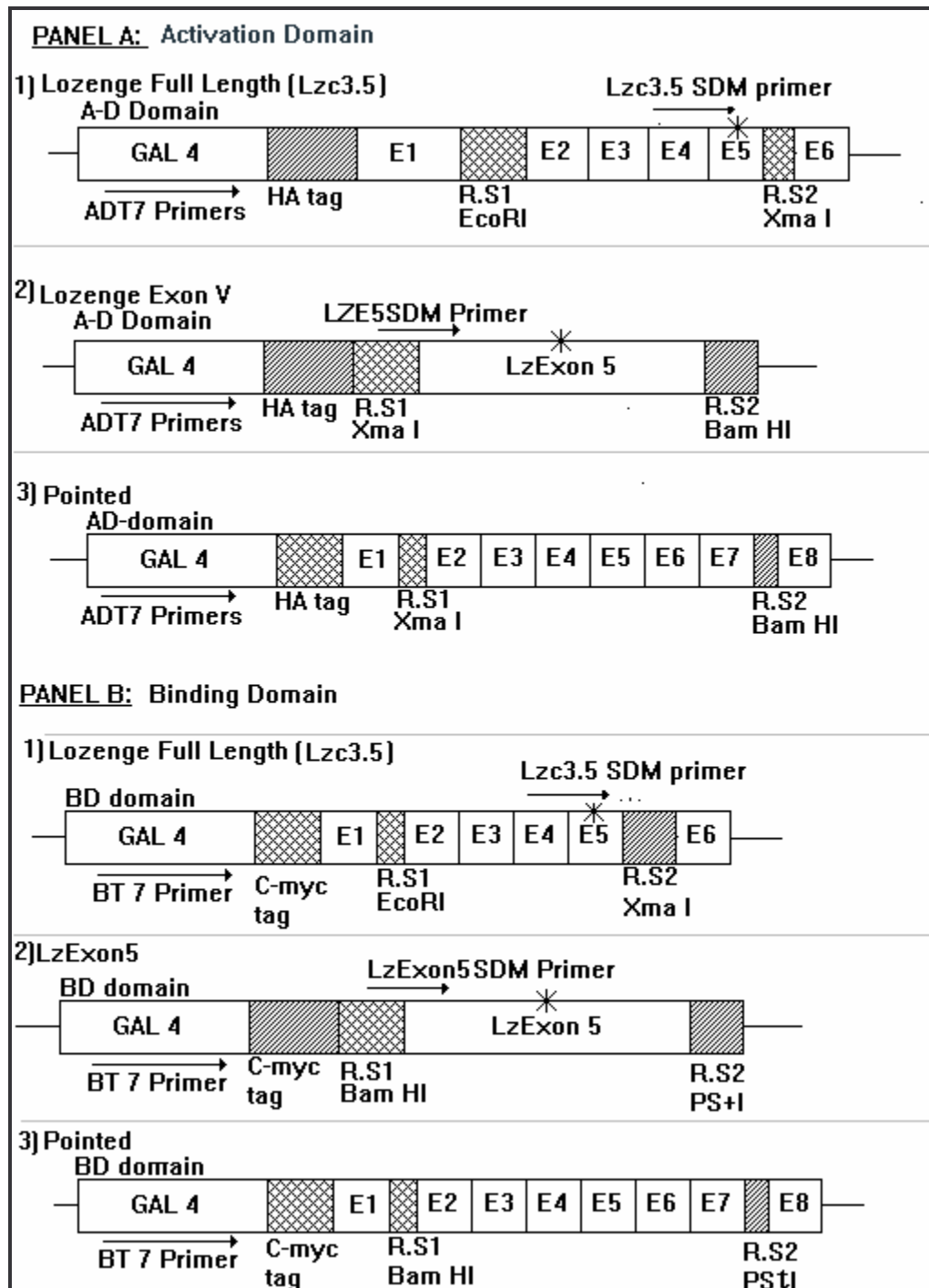
Each sample was checked by PCR amplification followed by suiTable restriction digestion. About (300-400ng) of each construct samples was sent for sequencing to the Molecular Medicin Institute, Pittsburgh (<http://www.mmi.pitt.edu/dnaseq.html>).

A cryoprotected stock of each plasmid construction in *E. coli* cells was prepared, snap frozen and stored at -80 °C as described above.

Table 7: Primers used for sequencing yeast clones.

5'DNA-BD sequencing primer (BT7)	5'-TCA TCG GAA GAG AGT AGT AAC AAAG-3'	55.6°C
5'DNA-AD Sequencing primer(ADT7)	5'- CTA TTC GAT GAT GAA GAT ACC CCA-3'	55.4°C

Figure 6: Yeast clones that were generated to test for Lozenge-Pointed interaction: Panel A: All clones fused to the activation domain. Panel B: All generated clones in the binding domain. The corresponding wild types clones to each mutant clone were also generated. The (*) shows the site directed mutagenesis, the arrows are the primer that were used for sequencing.

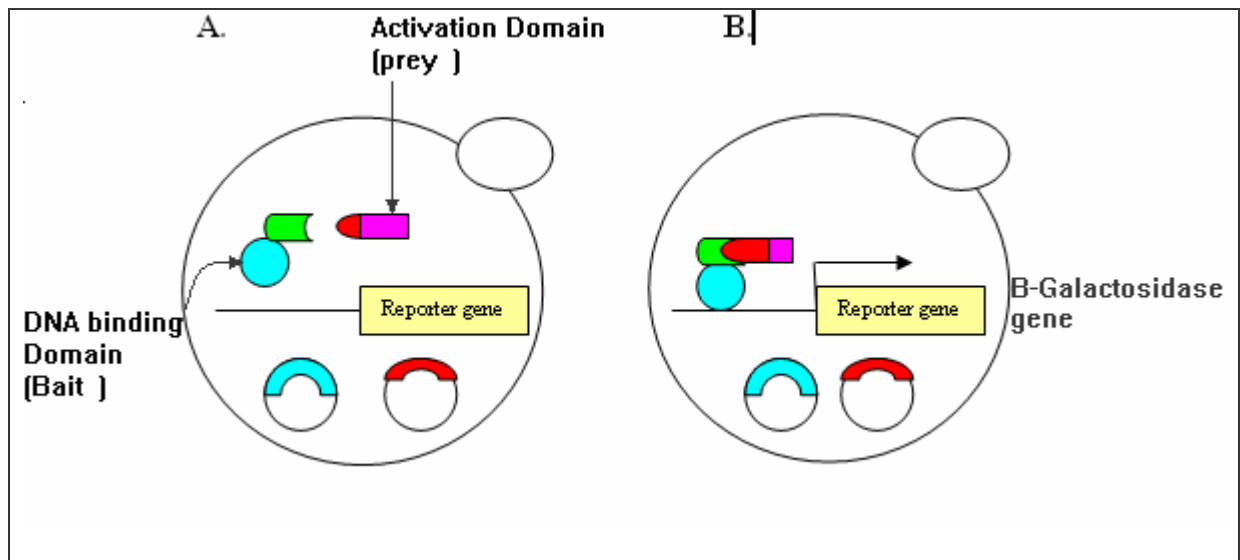


VII. Yeast two-hybrid System:

The yeast two-hybrid experiments were facilitated by using a packaged system, Matchmaker GAL4 Two-Hybrid System 3 by Clontech (cat #K1604-1). The test is based on the in vivo activity of the Gal4 protein, a transcriptional activator of the beta-galactosidase gene (Fig 7). The Gal4 protein has been broken in two parts, an activation domain and a DNA binding domain. The design purpose of this study was to determine if demonstrate that LZ protein can directly interact with PointedP2. So, full length LZ protein or LZ peptide exonV is fused to one or the other of the two Gal4 functional domains. The PNT2 protein is fused to the complementary Gal4 component as described above. Each fusion protein construction contains a c-myc or HA epitope tag. When the two Gal4 fusion protein components are transfected into the same yeast cell, the Gal4 protein becomes functional, and transcriptionally activates the beta-galactosidase gene, which is subsequently indicated by a color-changing reporter assay.

Figure 7:

Yeast two-hybrid system and gene fusion: The binding domain (cyan) is as a translational fusion with a gene encoding X protein (green) in one plasmid (Bait). The activation domain (purple) is also as a translational fusion with the gene encoding for the Y protein (red) (Prey).



1. Frozen strains recovery and preparation of working stock

plates:

The two yeast strains Y187 and AH109 provided with the kit were recovered from -80°C by streaking them in YPD agar plates, which were incubated at 30°C until yeast colonies reach $\sim 2\text{mm}$ in diameter. These plates can serve as working stock plate when stored 4°C . The AH109 yeast strain eliminates false positives by using three reporters-ADE2, HIS3, and MEL1 or (lacZ). The Y187 strain uses lacZ as a reporter gene.

2. Auto-activation check for individual plasmids constructs:

2-1. Transformation of yeast cells using LiAc protocol:

5 ml of YPD broth media was inoculated with 1 to 2 colonies and incubated with shaking overnight at 30°C. This culture is enough to inoculate 50ml culture to give an OD600 between 0.5-1. The cells were pelleted in 50 ml centrifuge tube at 3000xg (2500rpm) for 10 minutes. The media was poured off and 20 ml of sterile dionized water was added to wash and resuspend the cells. The tube was centrifuged again for another 10 minutes to remove the water.

The harvested cells are ready for transformation, the cells were suspended in 1 ml of 100mM lithium acetate (LiAc) and transferred to a sterile 1.5 ml microfuge tube. The cells were pelleted at top speed for 1 minute, LiAc was removed and the cells were suspended in a final volume of 500 μ l (400 μ l of 100 mM). The cell suspension was vortexed and 50 μ l samples were pipetted into a labeled microfuge tubes corresponding to each transformation sample. The cells were pelleted and the LiAc was removed, and the following ingredients were added carefully in the order listed:

- 240 μ l of PEG (50% w/v).
- 36 μ l of 1 M LiAc.
- 5 μ l of single-stranded carrier DNA (10mg/ml).
- 50 μ l of DI H2O and plasmid DNA (0.1-1 μ g)

In order to check the plasmid constructs on –leu/ -trp plates, each plasmid clone was co-transformed with an empty plasmid that contains the opposite auxotrophy. Each tube was vortexed vigorously until the cell pellets were completely mixed. The tubes were incubated at 30° C for 45 minutes and followed by a heat shock at 42°C water bath for 23 minutes.

The cells were centrifuged at 14000rpm for 1 minute to remove the transformation mix. 500 μ l of DI H₂O was added to the tubes and the pellet was suspended by pipetting gently. 200 μ l of each transformation was used for plating onto the appropriate selective media. The plates were incubated at 30°C until appearance of colonies. Two procedures were used to check the self activation of individual clones.

2.2. Streaking colonies in –leu /-trp X- α - gal indicator plates:

Colonies from each sample was streaked on patches on –leu/-trp X- α - gal indicator plates to detect the reporter gene estimated by the secreted α -galactosidase enzyme into the media encoded by the MEL1. The plates were incubated at 30° C for 2-3 days and checked for any possible appearance of the blue color.

2-3. β -galactosidase liquid assay: Quantitative method:

The yeast β -galactosidase assay kit (Cat # 75768) was used to check for auto-activation of lacZ reporter gene for each individual clone. Individual colonies taken from fresh -Leu/ -Trp plates were assayed for self-activation according to the manufacturer instructions.

3. Co-transformation of yeast strains for protein-protein interaction:

Yeast colonies were grown in 5 ml YPDA broth media overnight at 30°C shaking at 250 rpm and served to inoculate 50 ml of YPDA broth media. The colonies were grown until mid-log phase and then prepared for transformation depicted earlier. A molar ratio of 1:2 of prey (AD-domain) to bait (B-D domain)

was used for the co-transformation. Each transformation reaction was plated in SD minimal media X-alpha-gal –Leu /-Trp indicator plates. The plates were incubated at 30° C for 2-3 days. Positive clones appear as blue colonies, and they were streaked on patches in X-alpha-gal –Leu /-Trp indicator plates to confirm protein interaction.

4. Qualitative and quantitative assay for protein-protein interaction: β-galactosidase liquid assay:

After co-transformation cells were grown in –Leu/ -Trp liquid media for 2-3 days with shaking at 250 rpm. The OD was checked periodically, until the cells reach a mid-log phase (OD₆₀₀ of 0.5 and 0.8). At OD₆₀₀ of 0.5 and 0.8 each sample was made in triplicate and assayed for β-galactosidase activity. A volume of assay buffer was added to an equal volume of the Yeast Extract Reagent (Y-PER) to make the working solution. 350 μl of each test culture was transferred to three microfuge tubes and 350 μl of the working solution is added and the timer was started. The reactions tubes were incubated at 37°C in a water bath until the color change was observed. A blank tube was prepared by adding 350 μl of the culture medium (no cells) to 350 μl of the working solution (WS) and to 300 μl of the stop solution. This experiment also included both positive and negative controls the same mentioned above. When the yellow color appeared, 300 μl of the stop solution was added, the tubes were vortexed for 15 seconds, and the total incubation time was recorded. The tubes were centrifuged and the supernatant was transferred to a cuvette and the absorbance at OD₄₂₀ was measured. The activity of beta-galactosidase was calculated.

Preparation of media and reagents:**Site Directed Mutagenesis and Subcloning****LB Agar (per liter)**

10 g NaCl
10 g Tryptone
5 g Yeast extract
20 g Agar
Added deionized water to a final volume of 1 liter
Adjusted pH to 7.0 with 5 N NaOH
Autoclaved
Cooled to 55°C
Added 50 mg of filter-sterilized ampicillin/kanamycin
Poured into Petri dishes

NYZ+ Broth (per liter)

10 g NZ amine
5 g Yeast extract
5 g NaCl
Added deionized water to a final volume of 1 liter
Adjusted pH to 7.5
Autoclaved
Added the following filter-sterilized supplements prior to use
12.5 ml of 1M MgCl₂
12.5 ml of 1M MgSO₄
20 ml of 20% (w/v) glucose

SOB Medium (per liter)

20.0 g Tryptone
5.0 g of NaCl
Autoclaved
Added 10 ml of 1 M MgCl₂ and 10ml of 1 M MgSO₄ /liter of SOB medium prior to use.

SOC Medium (per liter)

SOB medium
Added 1 ml of a 2 M filter-sterilized glucose solution and filter sterilized.

Yeast Two Hybrid

YPDA (per liter)

960 ml d H₂O
50 g YPD
15 ml of 0.2% adenine hemisulfate
20 g agar added for plates
Deionzed water to make 1L
Adjust pH to 6.5
Autoclaved for 30 minutes
Pour plates

SD Medium (per liter)

Add 800 ml dH₂O to to a 2 liter flask with a stir bar.
20 g of dextrose
1.7g of YNB salts (Vogt lab synthetic mix)
1 ml of 1 mg /ml liquid solution of trace components
5 g of ammonium sulfate
10 ml inositoo-free vitamin stock solution (100X)
7.5 ml inositol stock solution (10mM)
0.64 g/ L of drop-out powder

Autoclaved for 30 minutes

Let the media cool down to 55 C than add X- α -gal

Poured plates

For single transformation the amino acid not carried by plasmid was also added to media. (200 mg/L for Trp and 1000 mg/L for Leu)

X- α - gal Solution

20 mg /ml in DMF

IV. Results:

A. Site directed mutagenesis

From previous research it was found that lozenge ExonV encodes a possible binding sites that serve for protein-protein interaction. Site directed mutagenesis was used to create mutants of lozenge full length (*lzc3.5 SDM*), and *lozenge exon V* (*lze5SDM*) in the helix to produce a kink and disrupt the Ets binding sites transcription factor Pointed. Primers were designed to mutate both the wild type lozenge full length and lozenge Exon V that change Leucine to a Proline (L439P) in the alpha helix of Exon V. This mutation is supposed to introduce a kink in the α -helix and consequently disrupt its secondary structure. Proline is a cyclic amino acid, due to cyclic binding of the three-carbon side chain to the nitrogen of the backbone; proline lacks a primary amine group (-NH₂). When proline is in a peptide bond, it cannot donate a hydrogen bond to stabilize an alpha helix or a beta sheet due to the lack of the hydrogen on the alpha amino group. It is often said, inaccurately, that Proline cannot exist in an alpha helix. When proline is found in an alpha helix, the alpha helix will have a slight bend due to the lack of the hydrogen bond. The same stretch of amino acid sequences of both the wild type that contains Leucine and the mutated alpha helix that contains Proline are computed using the software Phyre, to predict the secondary structure of the alpha helix.

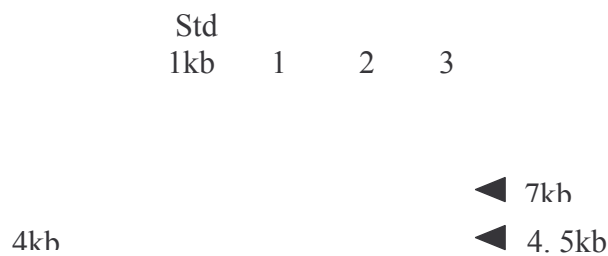
(http://www.sbg.bio.ic.ac.uk/phyre/qphyre_output/9f4336a1461b22d6/summary.html). The outputs of different softwares agree that the wild type stretch of amino acids sequence is an alpha helix (Figure 17 A). On the hand, when leucine is substituted by a proline the secondary structure of the alpha helix is completely

distorted (Figure 17 B). The predicted secondary structure of the alpha helix is very limited because the stretch of the amino acid does not show the complete folding of the whole protein.

Using the designed primers, the mutation was introduced by PCR technique. A 1% agarose gel was used to check the PCR product size; Whitescript 4.5 Kb was used as control for the site directed mutagenesis. All samples including the control showed the right size (Figure: 8). The Whitescript 4.5 Kb was used to test the efficiency of mutant generation. This control plasmid contains a stop codon (TAA) at the position where a glutamine codon (CAA) would normally appear in the -beta-galactosidase gene. The oligonucleotide control primers create a point mutation that revert the (T) residue of the stop codon (TAA) in the beta galactosidase gene to a C residue to produce a glutamine codon (Gln, CAA). All PCR products samples and control were digested by Dpn I endonuclease to remove methylated and hemimethylated parental DNA templates and select for mutation containing synthesized DNA. PCR products of pCR blunt lozenge full-length (Izc3.5SDM) mutant and lozenge Exon 5 mutant (IzE5SDM) including the control were transformed in XL10-Gold ultracompetent cells provided with the kit. 250 µl of the transformation reaction of each sample was plated on LB- Kanamycine agar plates containing 80 µg/ml X-gal and 20mM IPTG. The pUC18 transformation control was used to check the efficiency of the transformation and identification of positive clones was done by blue/white screening of LacZ induction. All plates including the control showed a high mutagenesis and transformation efficiency. Each sample DNA was purified by miniprep and 600-700 bp were sequenced to check for the desired

mutation starting from the junction between exon 4 and 5 to the exon 6 for lozenge full length. On the other hand, 700 bp DNA sequence covered the whole length of Lozenge exon 5 clones showing the correct orientation, the reading frame and the desired mutation (Figure: 6).

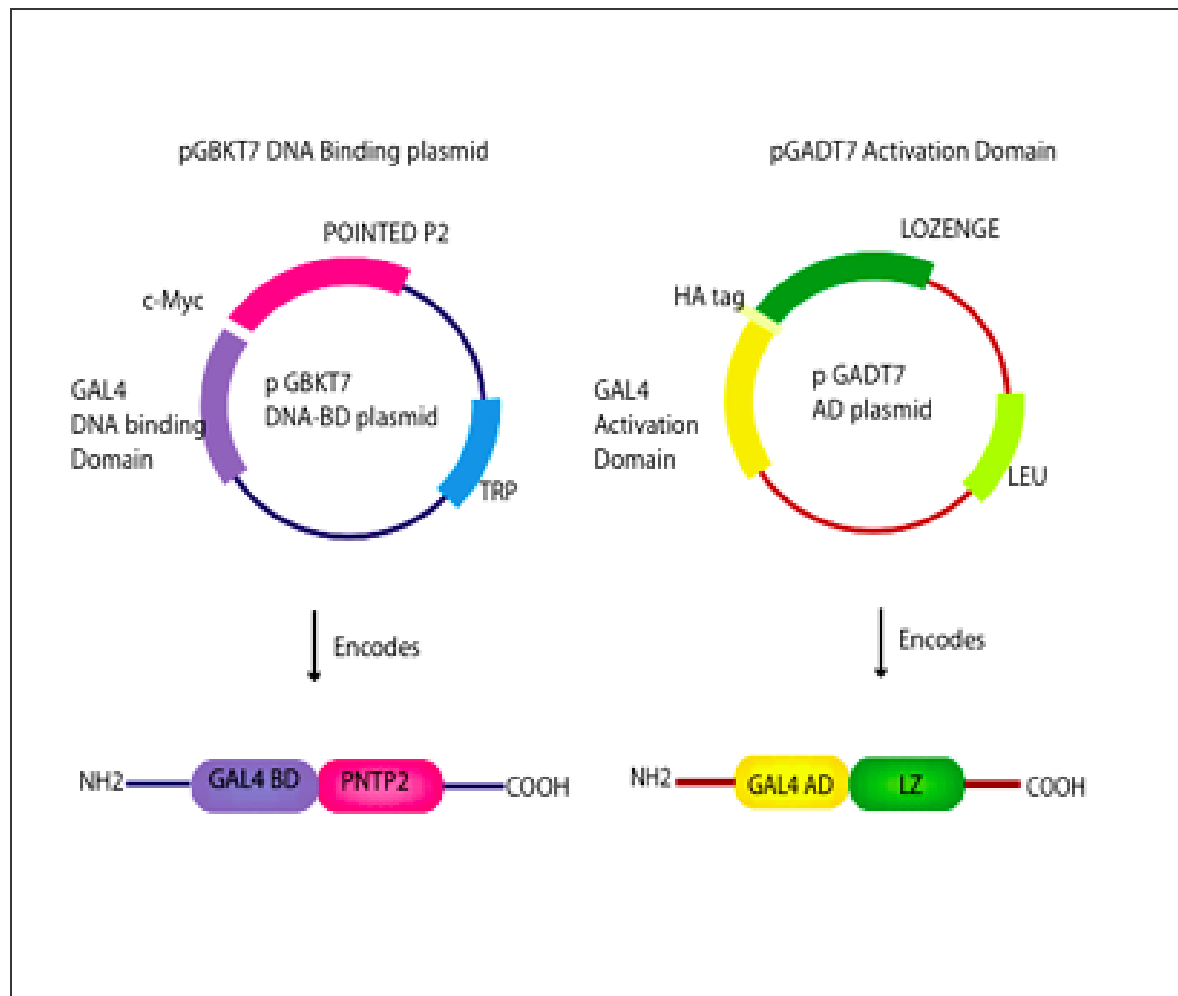
Figure 8: 1% agarose gel electrophoresis of the site directed mutagenesis:
Lane 1: shows site directed mutagenesis of lozenge c3.5 (3.5 kb) in pCRblunt vector (3.5kb), which gives 7kb PCR product. **Lane2:** shows site directed mutagenesis of lozenge Exon V (400bp) in pCR blunt vector (3.5 kb), which gives a 4 kb PCR product. **Lane 3:** The Whitescript 4.5 Kb mutagenesis control plasmid.



QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

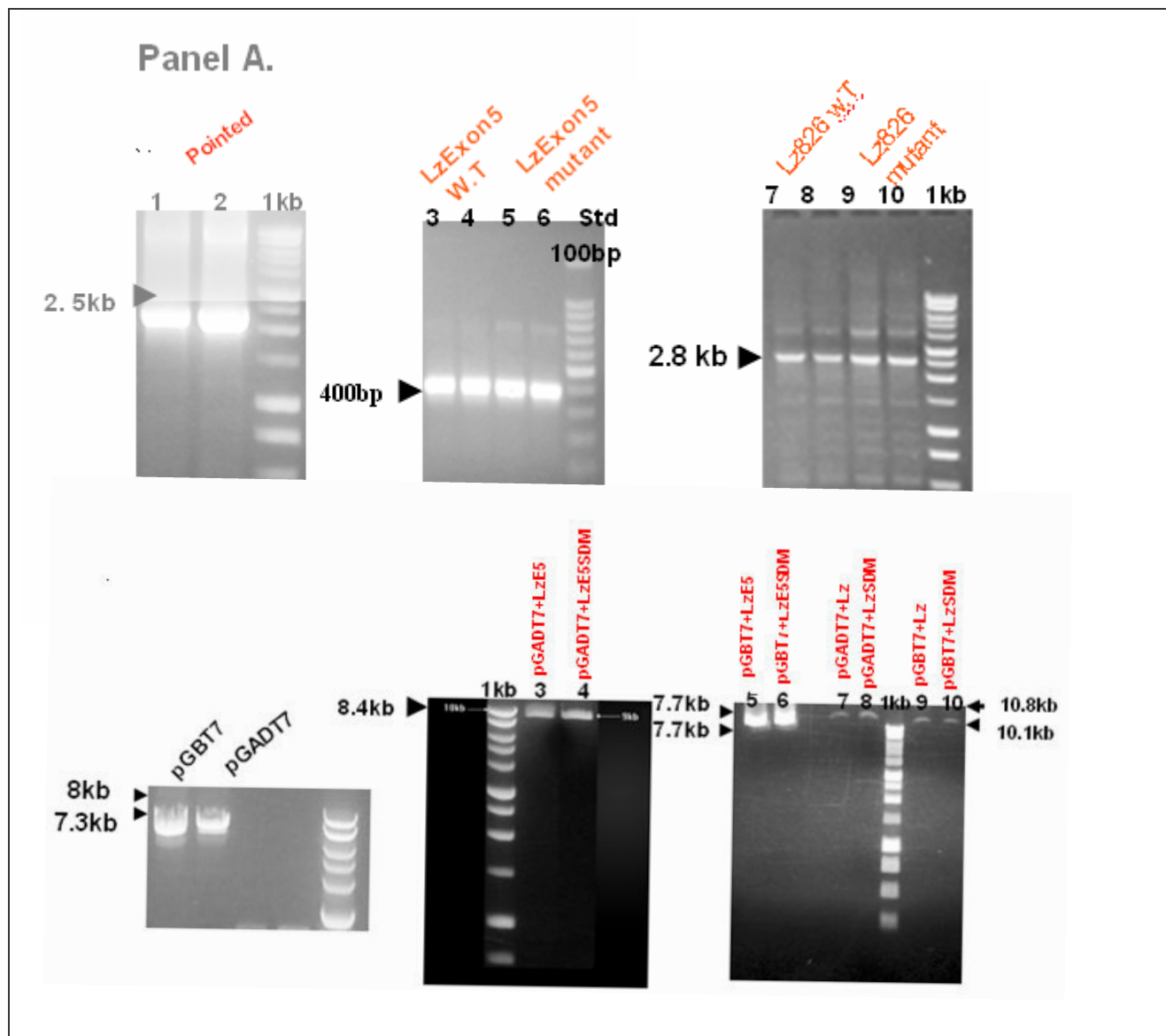
Yeast two hybrid system:

The intact Gal4 protein is a transcriptional activator that has two separate functions, a DNA-binding domain and an activating domain. It is possible to split the yeast *Gal4* gene in two parts - the activating domain and the binding domain and fuse one domain to a protein X and the other domain to a protein Y (Fields and song 1989). If the two chimeric proteins (X and Y) interact and bind one to the other, they can reconstitute a functional transcription factor that can activate the reporter gene (fig 7). The two reporter genes used in this experiment were the LacZ and MEL1. The lacZ gene is under the control of Gal4-responsive sequences, and it encodes the beta-galactosidase protein that uses X- gal as a substrate used in blue/white colony selection. The MEL1 is a yeast gene that encodes for α -galactosidase protein, which is a secreted enzyme that can be assayed directly on X- α - Gal indicator plates, using blue/white screening.

Figure 9: Vectors used in the two-hybrid system and the fusion proteins.

1. Fusion genes:

Lz mutant (*/zc3.5 SDM*) and Iz ExonV mutant (*E5 SDM*) clones and their corresponding wild type clones were amplified using primers that contain built-in restriction sites at the end of each DNA sequence (Table 3 and 4). The same restriction sites were found at the appropriate orientation in the vector and lozenge inserts were used to digest and linearize the yeast vectors pGBKT7 and pGADT7 that contain binding domain and the activation domain respectively. The purpose was to produce a chimeric protein that contains a fusion of both yeast GAL4 gene and lozenge inserts in frame (Figure 9). Samples were amplified and restriction sites were introduced using the PCR technique. 1 % agarose gel revealed the correct size of each sample DNA amplified (Figure 10: panel A).

Figure 10: Subcloning of gene of interest in Yeast vectors.

Panel A: Compatible restriction sites were introduced by PCR and each product was checked in 1% agarose gel. **Lane 1 and 2:** Pointed (P2), **lane 3 and 4:** Lozenge exon V (LzE5), **lane 5 and 6:** Lozenge Exon 5 SDM (LzE5 SDM). **Lane 7 and 8:** Lozenge full length Lzc3.5, **lane 9 and 10:** mutant Lozenge full length (Lzc3.5 SDM) **Panel B:** Linearization by single restriction digestion of yeast lozenge clones to check sizes: **Lane 1:** pGBT7-BD domain plasmid without insert, restriction digestion XmaI, **lane 2:** pGADT7-AD domain without any insert, restriction digestion XmaI, **lane 3-4:** LzE5 and LzE5 SDM in pGADT7-AD vector, XmaI digestion, **lane 5-6:** LzE5 and LzE5 SDM in pGBT7-BD vector, PstI digestion, **lane 7-8:** Lzc3.5 and Lzc3.5 SDM in pGADT7-AD vector, XmaI digestion. **Lane 9-10:** Lzc3.5 and Lzc3.5 SDM in pGBT7-BD vector, XmaI digestion.

Sequential restriction enzyme digests of both *LzExonV* wild type (*LzE5*) and *Lz Exon V* mutant (*LzE5 SDM*) were done efficiently after amplification, and the digested DNA were gel purified to remove any trace of buffer and enzymes. On the other hand, the purified PCR products Pointed (*Pnt P2*) and lozenge full length, its mutant (*lzc3.5*) and (*lzc3.5 SDM*) with the appropriate restriction sites were ligated to another pSC-B vector to ensure a complete digestion before to be ligated to the yeast plasmids. Restriction digestion of lozenge full length (*lzc3.5*) and its corresponding mutant were first digested by *Xma*I. 0.8% agarose gel revealed two bands, one band corresponded to the *Xma*I restriction site that was introduced by PCR amplification and the other was preexistent in the vector (Figure 11-A). The double digestion *Xma*I/ *Bam*HI of Pointed DNA showed 3 bands (Figure 11-B) because *Bam*HI restriction site originally preexisted in the pSC-B vector in addition to the other *Bam*HI restriction site that was introduced by PCR amplification (fig12 A and B).

Both digested yeast plasmids pGBKT7 and pGADT7 were dephosphorylated with a phosphatase to avoid recirculazation during ligation (fig 10-B). A 1: 3 molar ratio vector / insert were used in the ligation reaction. DNA samples were inserted to both pGBKT7 and pGADT7 in two different combinations as listed in Table below (Table 8). After transformation in H101 competent cells, high transformation efficiency was used. After DNA purification, samples were checked by restriction digestion by the appropriate restriction enzymes (Table 4), and by PCR using the same primers used previously for amplification. All samples revealed expected sizes in 0.8% agarose gel (Figure 10: panel B), a total of 10 chimeric genes were made and were sequenced,

revealing that all *Drosophila* DNA inserts were in proper reading frame with the yeast GAL4 protein.

Table 8: List of the two combinations of clones that were designed for yeast two-Hybrid -system.

Yeast plasmid pGBKT7 (Binding domain+ Trp)	Yeast plasmid pGADT (Activation domain +Leu)
Lozenge wild type (Lzc3.5) Mutant Lozenge (Lzc3.5 SDM) Lozenge Exon V (E5) Mutant Lozenge Exon V (E5 SDM) Pointed (P2)	Lozenge wild type (Lzc3.5) Mutant Lozenge (Lzc3.5 SDM) Lozenge Exon V (E5) Mutant Lozenge Exon V (E5 SDM) Pointed (P2)

Figure 11: 0.8% agarose gel showing restriction digest of both Lozenge full length (Lzc3.5), mutant lozenge full length (Lzc3.5 SDM) and Pointed (P2).

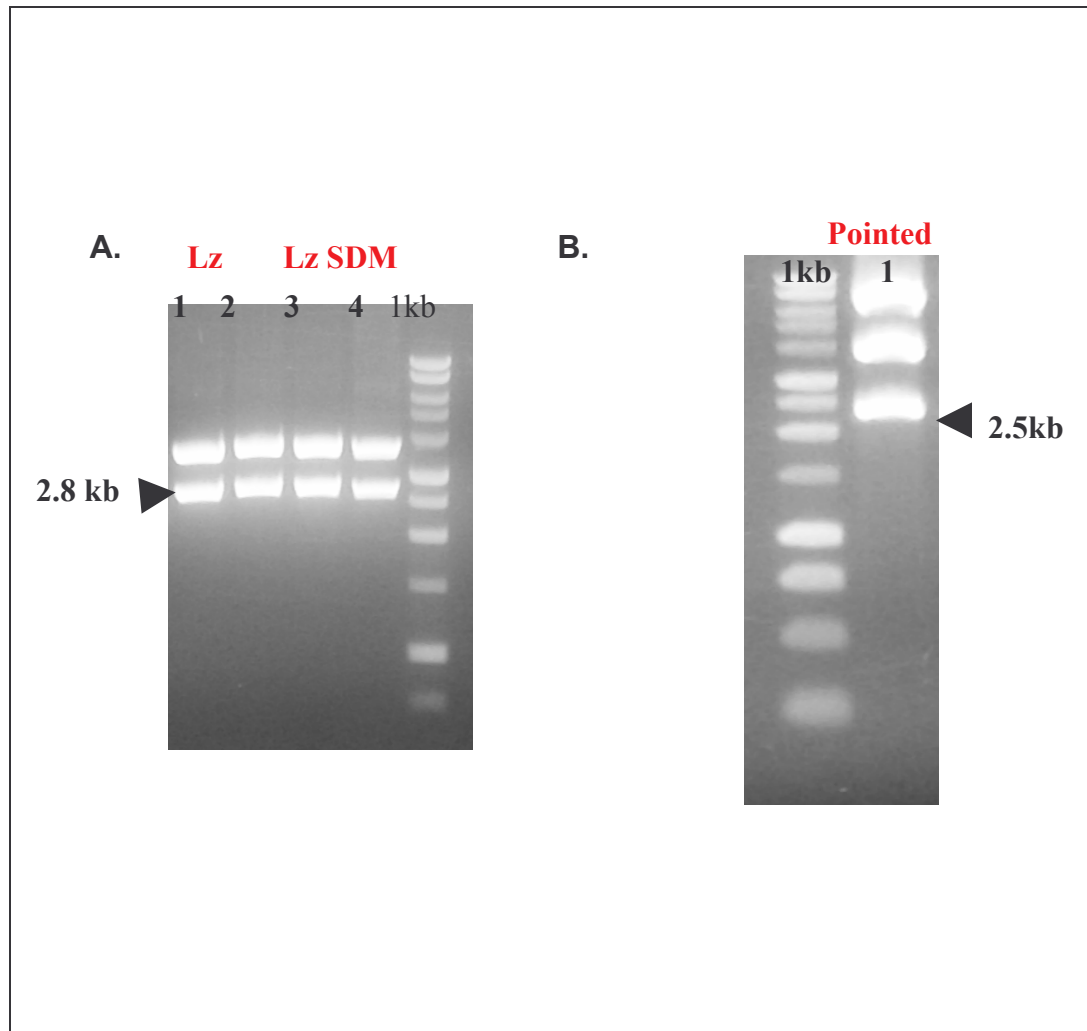
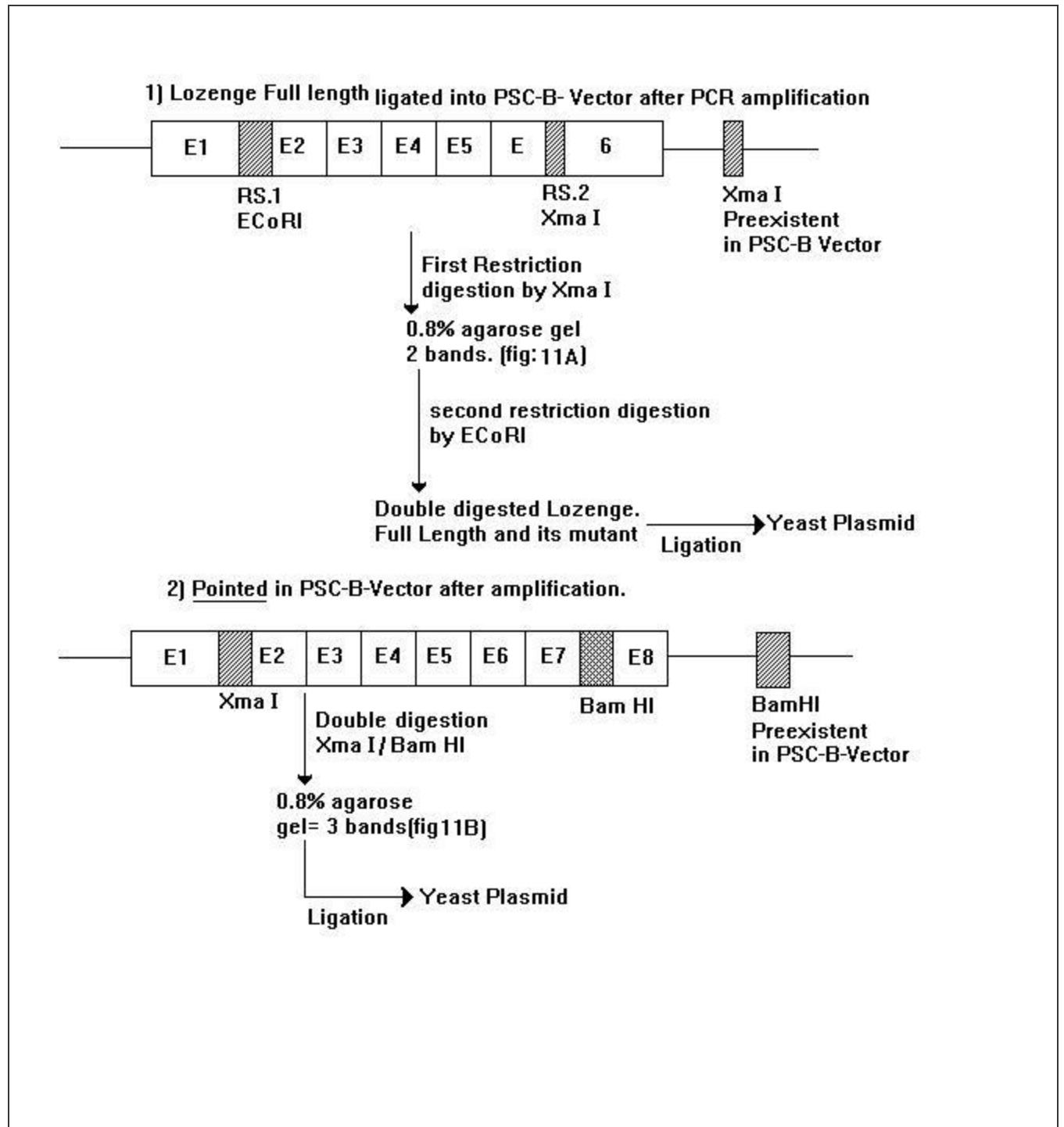
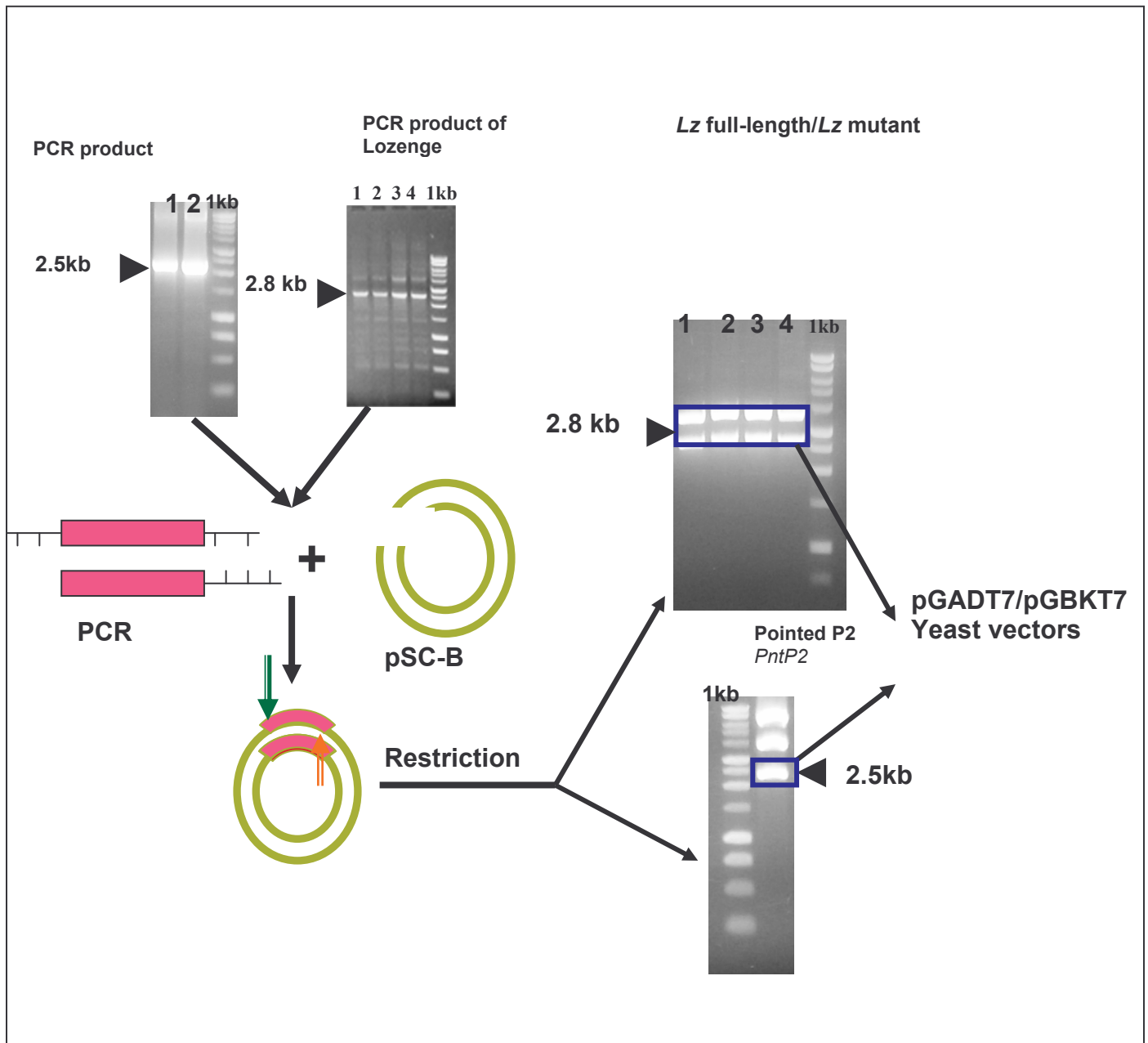


Figure A: lane1-2: Restriction digest of Lzc3.5 and Lzc3.5 SDM (2.8kb) by XmaI, the obtained two bands correspond to the digestion of the two restriction sites of XmaI. One site is preexistent in the pSC-B vector at the position 159; the other restriction site is introduced by PCR. **Figure B:** Restriction digest of Pointed (P2) (2.5kb) by XmaI / BamHI in psc-B vector.

Figure 12: Summary of steps employed for preparation of lozenge full-length and pointed used before ligation into the yeast vectors.

A.



B.

2. Checking for auto-activation:

Auto-activation was checked for a successful assay. Y187 and AH109 yeast strains were recovered from the -80°C and grown at 30°C for 3-4 days in YPDA plates. Colonies about 2-3mm size were used to inoculate 5 ml of YPDA broth media. The grown 5ml culture was used to inoculate 50 ml YPDA broth media.

2.1 Auto-activation using liquid assay:

Individual sample clones (auxotrophic for Trp and Leu) were transformed into yeast strains using Lithium acetate procedure. All pGADT7 clones were plated in SD - Leu media supplemented with Trp. On the other hand, sample clones in pGBKT7 plasmid were plated in SD -Trp supplemented with Leu. Each transformation reaction was made on duplicate, and single colonies were picked from each plate and assayed for beta-galactosidase activity.

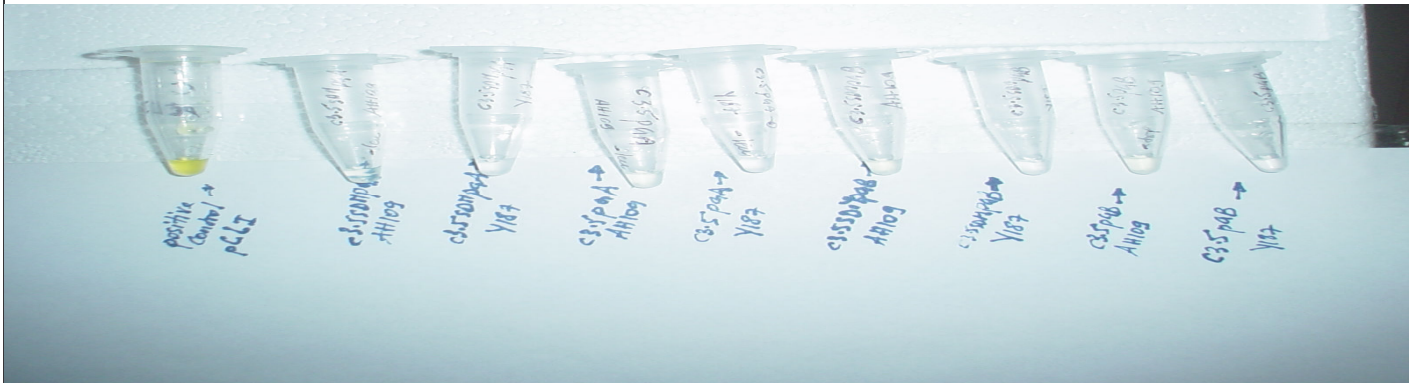
A portion of the colony from a solid media is suspended in an equal mixture of Yeast protein extraction reagent (Y-PER) and β -galactosidase assay buffer. After an incubation period, the positive control PLC 1 that encodes the wild type yeast GAL4 transcription factor turned yellow and no sample clones showed any yellow color (Figure 13 panel A). The solution turns yellow in the positive control from the hydrolysis of the colorless O-nitrophenyl- β -D-galactopyranoside (ONPG) to O-nitrophenol (ONP), which has a yellow color and galactose in a mildly alkaline solution.

2.2 Auto-activation check using X- α -galactosidase indicator plates:

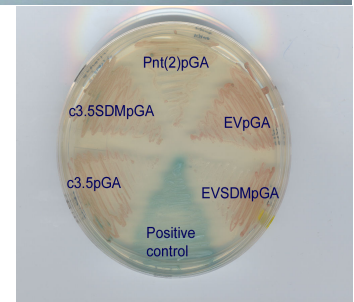
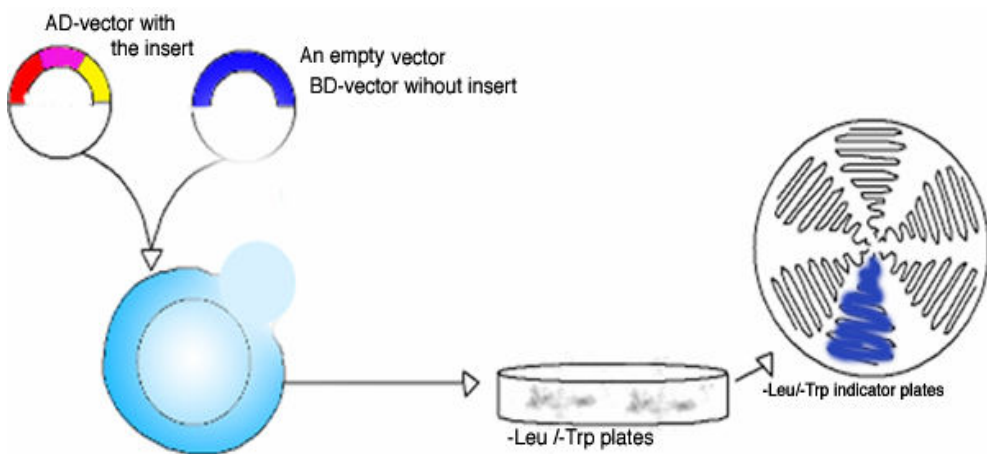
Samples clones in the activation domain and in the binding domain were checked for self-activation in X-alpha- galactosidase indicator plates to survive the nutritional selection media. Each sample clone in the binding domain was co-transformed with an empty cloning vector that has an activation domain and *vis-versa* for the set of the other clones. All transformation reactions samples were plated in X-alpha-gal plates, and incubated at 30°C for 3-4 days. All plates were screened for blue/white colonies. Colonies were taken from the plates and streaked in patches in X-alpha-gal plates after a period of incubation; they showed no activation of MEL1 reporter gene except for the PCL1 positive control that revealed a blue color in the media plate. Therefore, the sample clones by themselves cannot activate the reporter gene (Figure: 13 panel B).

Figure 13: Checking for auto-activation of yeast sample clones: **Panel A:** Sample clones transformed in both strains were checked for auto-activation using β -galactosidase liquid assay. **Panel B:** Yeast clones checked for auto-activation in X-alpha-Leu /-Trp indicator plates.

PANEL A:



PANEL B:



3. Co-transformation:

Sequential transformation of lozenge-Pointed DNA previously showed toxicity for the yeast cells and it was unsuccessful for detecting Lz-PNTP2 interaction (Singn's 2004). Therefore, this procedure was never assayed, and simultaneous transformation was used instead. A pGADT7-T+pGBKT7-53 and pGADT7-T+pGBKT7-lam plasmids were used as positive and negative controls respectively (Table 9). 200 μ l from each co-transformation reaction was plated in X-alpha-gal Leu /-Trp indicator plates. Colonies took 3-4 days of incubation period to appear, they grew at a slow rate and they were small in size. Colonies appeared blue and white on the indicator plates.

Table 9: Control plasmids used for yeast two-hybrid system.

Plasmids	-Leu / -Trp indicator plates	
pGADT7-T + pGBKT7-53	-Leu -Trp	Positive control
pGADT7-T + pGBKT7-lam	-Leu -Trp	Negative control

3-1- Reporter gene detection:

A failure to detect the activity of the reporter gene for β -galactosidase for several trials using the colony filter lift assay, prompted me to use different approaches to detect protein-protein interaction:

3.1.1. X- α -gal indicator plates:

After co-transformation blue colonies were picked from each plate and streaked in patches in X- α -gal –Leu /-Trp indicator plates to confirm the protein-protein interaction. The plates were grown for 3-4 days, and the appearance of blue color was checked periodically. All samples revealed a blue color, which includes the positive control (Figure 15) except the negative control and the mutant Lozenge Exon V. This technique was assayed three times in two different combinations listed in the Table 10).

3.1.2. Liquid assay:

After co-transformation of Lz-Pointed clones as described in lithium acetate transformation (Table 10). 200 μ l of each transformation reaction was grown in –Leu/-Trp liquid media for 2-3 days at 30° C incubator with shaking at 250 rpm until they reached an OD600 of 0.5 and 0.9. A 350 μ l sample of each reaction was used to assay the β -galactosidase activity by appearance of the yellow color after a period of incubation at 37°C. A pGADT7-T+pGBKT7-53 and pGADT7-T+pGBKT7-lam were used as positive and negative controls respectively showed color as expected (Figure 16).

Figure 14: Reporter gene constructs in yeast strain AH109, the two reporter genes are under the control of MEL1 UAS sequence and the MEL1 promoter.

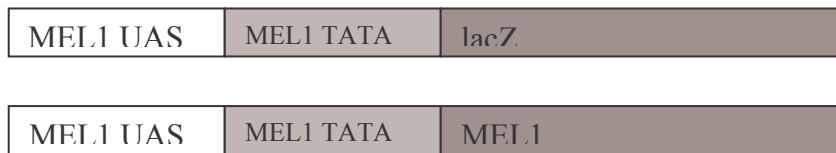


Table 10: Different combination of yeast clones used for co-transformation and assayed for yeast two-hybrid system.

Sample inserts in pGBKT7-Binding domain (Bait)	Sample inserts in pGADT7 -Activation domain (Prey)
Pointed	Lozenge full length (Lzc3.5)
	Mutant lozenge full length (Lzc3.5 SDM)
	Lozenge Exon V (LzE5)
	Mutant lozenge Exon V (LzE5 SDM)
Lozenge full length (Lzc3.5)	Pointed
Mutant lozenge full length (Lzc3.5 SDM)	
Lozenge Exon V (LzE5)	
Mutant lozenge Exon V (LzE5 SDM)	

4. β -galactosidase quantitative assay:

A quantitative assay for each combination of Lz-Pnt clones was used to assess the expression of the reporter gene by revealing β -galactosidase activity. Three replicates tubes of each sample were assayed for β -galactosidase activity at the $OD_{600}=0.5$ and 0.9 , and their corresponding absorbances at (OD_{420}) were measured as well. The mean of the absorbance of the three tubes corresponding to each OD were used in the calculation of the beta-galactosidase activity in the following equation (Ref).

$$\frac{1,000 \times A_{420}}{t \times V \times OD_{600}} = \beta\text{-galactosidase activity}$$

t = time (in minutes) of incubation

v= Volume of cells (ml) used in the assay

This experiment included both positive and the negative controls and they were as expected (Figure 16). All samples showed a low β -galactosidase activity comparing them to the positive control, which is about 10 fold higher (Table 11). In AH 109 yeast strain the level of lacZ expression is under the control of the weaker MEL1 UAS consensus sequence (Figure 14).

Table 11: Quantitative liquid assay by measuring β -galactosidase activity of the indicated sample clones in pGADT7-AD domain+ Pointed in pGBKT7-BD domain: AH109 yeast cells were used for the co-transformation. In **A and B:** β -galactosidase activity was calculated at the $OD_{600}=0.5$ and 0.8 respectively after 72 hours of cell growth.

A. β -galactosidase activity calculated at $OD_{600}=0.5$ of cell growth.

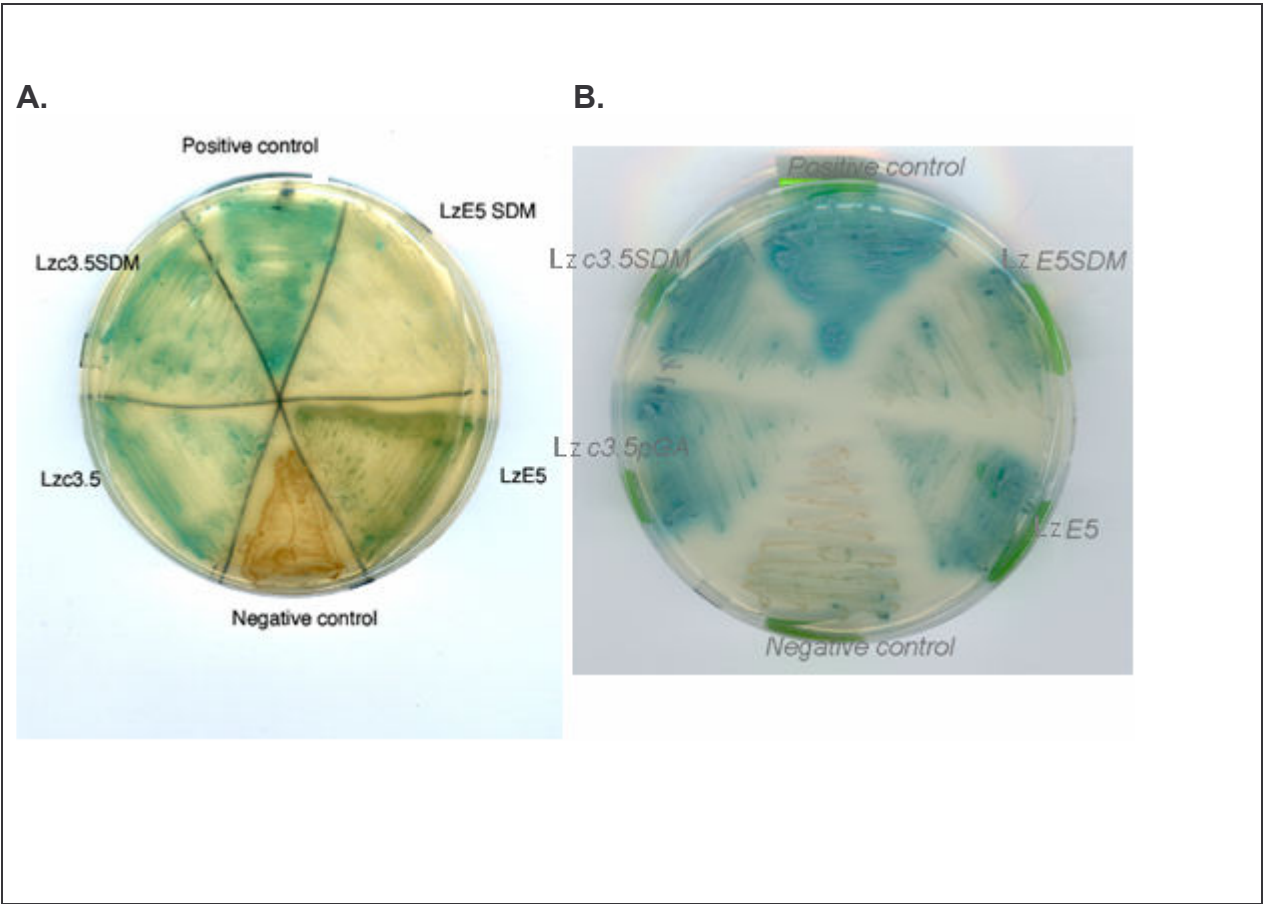
Yeast clones	LzE5	LE5SDM	Lzc3.5	Lzc3.5 SDM	Positive Control	Negative Control
OD_{600}	0.541	0.619	0.555	0.581	0.662	0.855
Incubation time (min)	106	106	106	90	30	40
Absorbance (A_{420})	0.051	0.101	0.127	0.119	0.325	0.029
β -gal activity	2.541	3.94	6.16	6.50	46.75	2.436

B. β -galactosidase activity calculated at $OD_{600}=0.8$ of cell growth

Yeast clones	LzE5	LE5SDM	Lzc3.5	Lzc3.5 SDM
OD_{600}	0.818	0.832	0.729	0.726
Incubation time (min)	100	80	80	90
Absorbance (A_{420})	0.104	0.126	0.150	0.156
β -gal activity	3.027	5.408	7.348	6.821

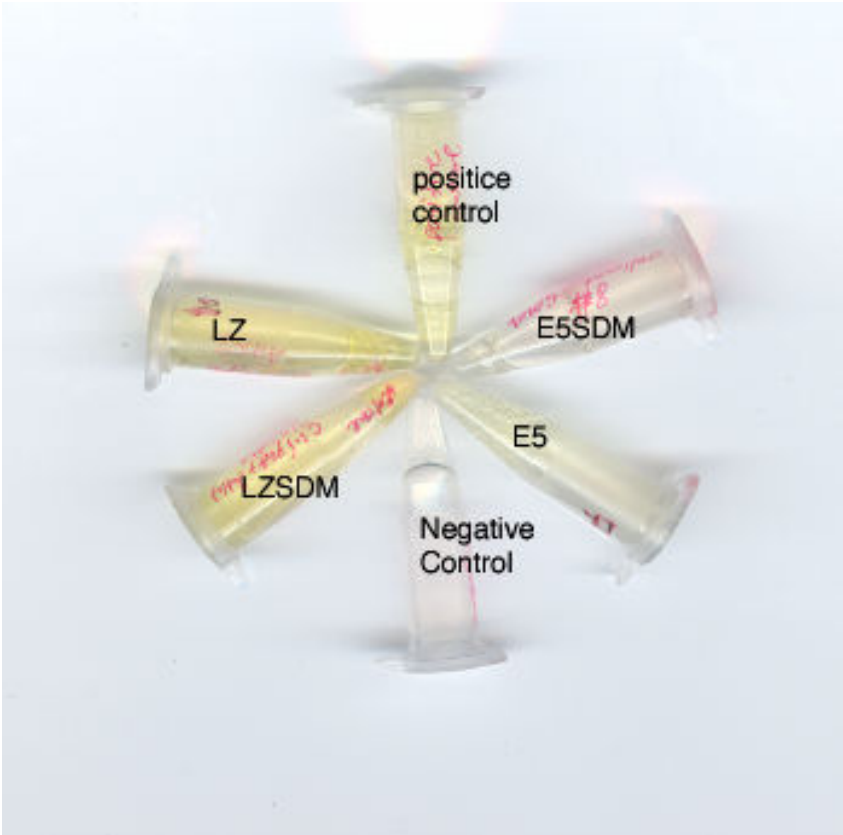
Figure 15:

The Table shows the strength of Pointed-Lozenge protein interactions according to the blue color intensity of the secreted α -galactosidase in the indicator plates. The (+ sign) means positive interaction with six plus means the most positive (strong protein-protein interaction), the (- sign) means negative interaction with six minuses means the most negative (no protein-protein interaction). Plate **A** and **B** are two separate co-transformations for the same sample clones plated in X- α -gal–Leu/-Trp indicator plates.

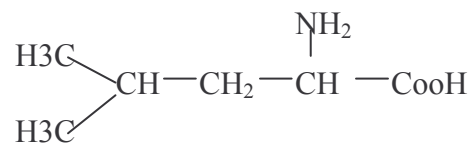
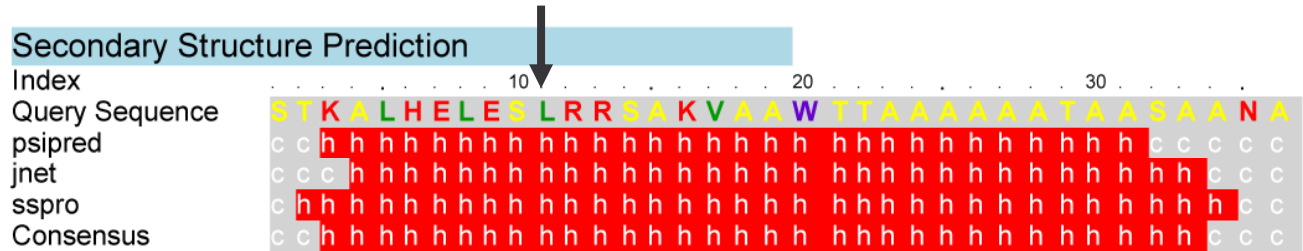
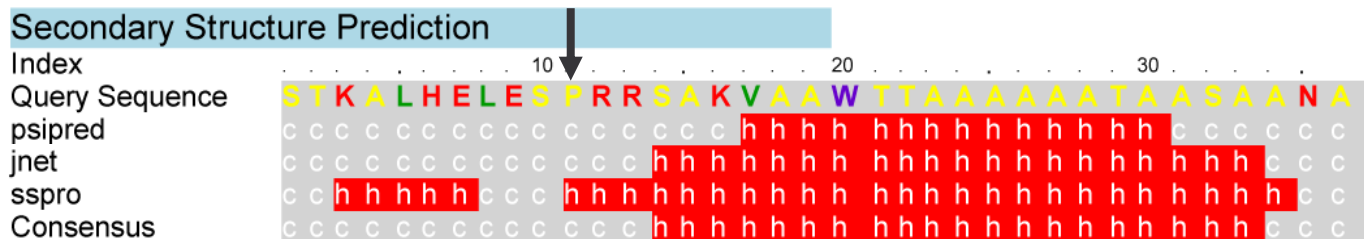


Clones	Positive control	Negative control	Lzc3.5	Lzc3.5 SDM	Lz Exon5	LzExon 5 SDM
The interaction strength	++++++	-----	+++	+++	++	+-

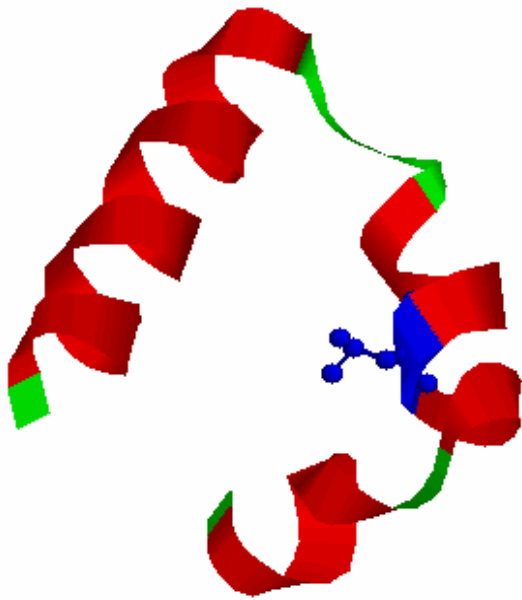
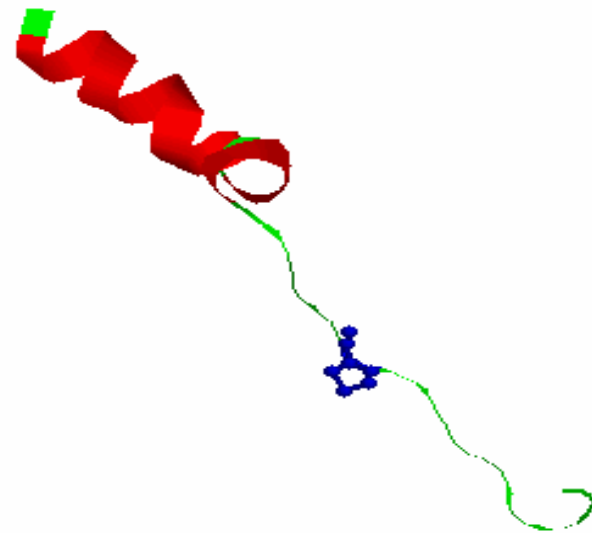
Figure 16: Checking protein-protein interaction by using β -galactosidase liquid assay.



Clones	Positive control	Negative control	Lzc3.5	Lzc3.5 SDM	Lz Exon5	LzExon 5 SDM
The interaction strength	++++++	-----	+++	+++	++	+-

Figure 17: The predicted secondary structure of the alpha helix.**A.****Wild type alpha helix****Leucine****Mutant alpha helix****Proline**

http://www.sbg.bio.ic.ac.uk/phyre/qphyre_output/9f4336a1461b22d6/summary.htm

B.**A. Normal helix (Leu)****B. Mutant helix (Pro)**

V. Discussion:

This study presents an assessment of the physical interaction of the transcription factors Lozenge and Pointed. In particular, I have explored the protein protein interactions using yeast two-hybrid system.

Given that both Lozenge and Pointed proteins are needed for the transcriptional activation of *prospero* expression (Behan et al 2005 ; Figure 4), We have explored the possibility that the Lozenge and Pointed proteins interact first before binding to the DNA and activate the expression of *prospero*. Based on the sequence similarity between Lozenge and the Runx1 homologs of other species, we suspected that this protein-protein interaction is facilitated by an alpha helical domain found in lozenge exon 5.

The fundamental hypothesis is that Lz826 protein interaction with Pointed is facilitated only by the alpha-helix amino acids 428-467 without the interaction of additional putative binding sites reported for AML1. To test this hypothesis a mutant clone L429P encoding Lozenge full-length protein Lz826 and the peptide Lozenge Exon V were generated to see if a potential disruption of the alpha helix was sufficient to interrupt the lozenge-pointed interaction.

The yeast two-hybrid system was the first method to be employed to test for protein-protein interaction because of the in vivo aspect of the assay. This assay was not restricted to the artificial interaction of the in vitro assays such as performed by co-immunoprecipitation or electrophoretic mobility shift assay. Lozenge and Pointed variants were fused to the GAL4 Activation Domain and Binding Domain carried by the two yeast vectors. DNA sequencing analysis of the clones confirmed that all *Drosophila* DNA inserts were in reading frame with the yeast GAL4. The yeast two-hybrid was

assessed on the two combinations of Lozenge-Pointed clones (Table 10). Another critical step that determined the achievability of this assay was to check for auto-activation of the reporter gene for individual clones. Individual clones auxotrophic either for Leucine or for tryptophan was co-transformed with an empty cloning vector that contained the opposite selective nutritional marker and checked for self-activation. This approach was done to test the same clones for auto-activation and protein-protein interaction in the same X-alpha-gal -Leu /-trp indicator plates. All clones that were checked for self-activation did not show any blue color; therefore individual constructs could not activate the reporter gene by themselves.

Protein-protein Lozenge--Pointed interaction was assessed by activation of the reporter gene. Two reporter genes used in this experiment: the lacZ reporter gene encoded for beta-galactosidase, and the MEL1 reporter gene encoded for alpha-galactosidase. Both reporter constructs were under MEL1-UAS promoter in AH109 yeast strain (Figure 14). When yeast cells were co-transformed with lozenge and pointed DNA, cells grew at a very slow rate and they were very small in size. Why would this happen? One possible explanation is that ectopic expression of Lozenge and Pointed might be toxic and interfered with the yeast metabolism. If Lozenge and/or Pointed expression were toxic to the yeast cells, we might expect there to be homologous proteins in the yeast genome. However, sequence analysis of the yeast genome revealed the absence of *Runx-like* genes (Adya et al). However, the Ets protein family members are represented in the yeast genome and exhibit a conserved region in their N-terminal part called pointed domain (PNT). Alignment of this conserved region (PNT) revealed that it is related to a region present in *Polycomb* protein and in

cytoplasmic proteins involved in yeast sexual differentiation named SEP (yeast Sterility, Ets-related, Polycomb proteins) (Laudet et al 1999). Based on this, it is possible that the toxicity acted as a selection pressure against the Pointed clone and that over time, and the Pointed DNA was “kicked out” from the plasmid and only the selective marker was kept intact. Overtime the cells containing just one insert with an empty vector overcome the total cell growth. Previous work showed when a sequential transformation was performed, yeast cells grew in the selection media lacking the two amino acids Leu and Trp indicating that the two plasmids were present in the cell, but activity of lacZ reporter gene was not detected (Singh, 2004). It was also demonstrated that beta-galactosidase activity was not detected using the beta-galactosidase liquid assay for cells grown from an originally ongoing growth –leu/ -trp broth. In order to detect the lacZ reporter gene expression by liquid assay, the test has to be done immediately before the toxicity started its effect on yeast cells. For this reason, the X-alpha-gal indicator plates were chosen over the colony lift filter assay for detection of the reporter gene because the alpha-galactosidase was secreted directly onto the plates.

Both clones LZ and Pointed were co-transformed simultaneously and plated in X-alpha-gal indicator plates. Positive colonies appeared blue and have almost the same color intensity as the positive control. Blue colonies for each clone were streaked in patches in the X-alpha-gal indicator plates and they appeared again blue confirming protein-protein interaction (Figure 15). Blue colonies indicated positive interaction between LZ –PNT2 proteins. Mutant and wild-type Lozenge full-length clones interacting with pointed appeared blue and they have almost the same blue color intensity like each other.

Beta-galactosidase qualitative liquid assay for mutant and wild type lozenge full-length and Lozenge Exon 5 correlated with the results obtained in X-alpha-gal indicator plates (Figure 16). Disruption of the alpha helix secondary structure by L429P mutation in Lozenge full-length cannot give us a good understanding for the involvement of the alpha helix in the interaction because lozenge Exon 4-5 DNA binding sequences were not disrupted. For this reason, lozenge Exon 5 mutant and wild type clones were used to assess this approach. Colonies of Lozenge Exon 5 wild type showed an activation of MEL1 reporter gene by expression of alpha-galactosidase enzyme and appeared blue in X-alpha-gal indicator plates. Lozenge Exon 5 clone was able to carry out protein-protein interaction with pointed using the alpha helix. To assess that the alpha helix had a major contribution in lozenge Exon 5-pointed interaction, mutant lozenge Exon 5 clone was created by L429P mutation to produce a kink in the alpha helix. The mutant Lozenge Exon 5 clone showed no interaction with pointed (Figure 15-Table). One interpretation is that the Lozenge-Pointed interaction was disrupted because the alpha helix was perturbed. The X-alpha-gal expression was ranked as (-3) comparing it to the negative control, which was evaluated by (-6) (Table of Figure 15). A single proline mutation was not enough to entirely disrupt the secondary structure of the alpha helix. Presumably the substitution of more proline amino acids would disrupt the helix more profoundly and consequently mutant Lozenge Exon 5-Pointed protein interaction.

A quantitative assay was done to evaluate Lozenge-Pointed protein interaction by measuring the beta-galactosidase activity (Table 11). Each value of beta-galactosidase represented the mean of three tests done in the same condition and at the same time. The AH109 yeast strain was used, in which the lacZ reporter gene was

under the control of the MEL1 weak promoter (fig 9). Consequently, values represented in Table 11 were very low by comparison to the positive control, which showed about 10 folds higher activity. In mutant and wild type lozenge full-length activity of beta-galactosidase was detectable, but was still results were hard to interpret.

In conclusion, my experimental results show that the introduction of the proline amino acid in the middle of exon 5 alpha helical domain is sufficient to weaken the protein-protein interaction between Lozenge and Pointed. However, a single proline is not sufficient to completely obliterate the protein-protein interaction. Further analysis is warranted. A second observation is that the yeast cells appear to resist expressing the Lozenge and/or Pointed proteins, presumably due to some level of toxicity. This may be due to the Drosophila transcription factors innate capacity to influence gene expression or it may be due to these foreign proteins's capacity to bind with and influence the function of a wide range of other proteins.

VI. Future directions:

Yeast two-hybrid results of mutant clones showed that *lozenge* Exon 5 was essential for interaction with Pointed but one single proline was not able to disrupt completely the secondary structure of the alpha helix. Therefore, creation of mutant clones that have two- to three more proline mutations might deeply interfere with Lozenge-Pointed interaction. In addition, generation of mutant clones, which consist of a complete deletion of the exon 5 (appendix-procedure) would allow us to have a better understanding of lozenge-Ets1 interaction because of the disruption of all binding sites. Furthermore, germline transformation of flies with the mutant would allow us to directly test the capacity of the abnormal protein to function in the developing fly eye .

Lozenge is not only required for proper differentiation of *Drosophila*'s eye but also for other tissue such as the antennae, tarsal claws and pulvilli, spermathecae and blood cells (Green and Green 1956; Green 1990; Stocker et al.1993; Betterham et al. 1996; Lebestky et al. 2000). Therefore, lozenge interaction is not only limited to Ets transcription factors, but also more likely to other lozenge partner transcription factors that could down regulate the expression of other genes during development. The creation of a cDNA library from tissue to screen for additional protein-protein interaction using the actual lozenge clones would allows us to identify novel lozenge partner proteins.

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VIII. Appendix:

This procedure was taken from the International Journal of Life Science Methods vol.

38, NO.6, June 2005: Site-directed mutagenesis using pfU DNA polymerase and T4

DNA ligase. Yair Aderth, Fristen J. Cahapion, Tiedn Hsum and Vincent dammai.

Medical University of South Carolina, Charleston, SC, USA.

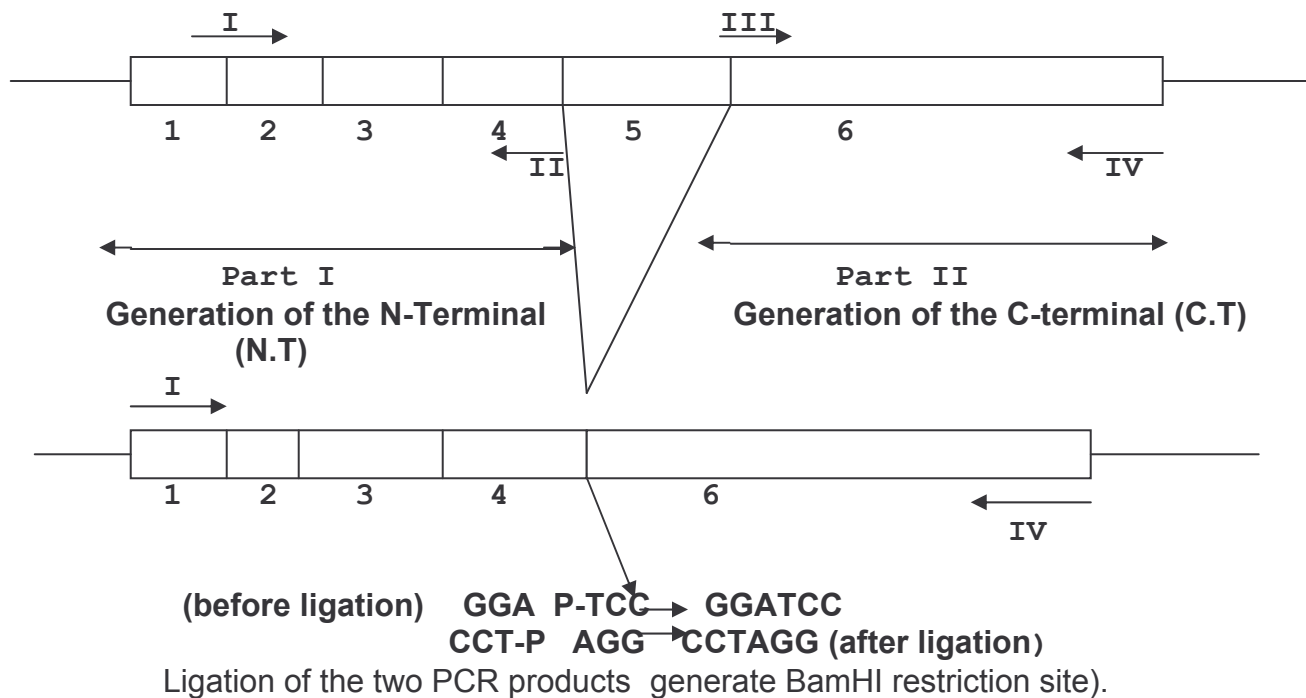
I modified some steps to optimize the experiment.

Table 1: Primers used for amplification to introduce EcoRI/XmaI restriction sites and to ligate samples to pGBKT7 and pGADT7 yeast vectors

Primer's name	Sequences	Tm	Length
Primer I	<u>GCGAATTC</u> ATGCATTTGCATCTCCTGGCGGCA (EcoRI)	69.5° C	32
Primer II	5' P-TTGTCTTGGAGCGGGGCTCGCGGGGTC 3'	71.3° C	27
Primer III	5' P- CAGCCGAAATGATGGGCGCCGGCGAATGGA 3'	70.4°C	30
Primer IV	<u>GCCCCG</u> GGTCAATAGGGTCGCCACACCT (XmaI)	72.2° C	28

Deletion of the exon 5: Strategy to delete Iz exon 5

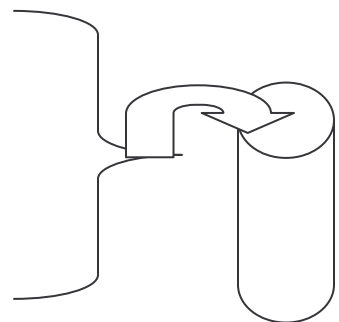
I. Restriction sites were introduced by amplification using both primers I and IV, which contain built-in restriction sites and the procedure is done on two steps. The two generated PCR products generate BamHI restriction site after ligation

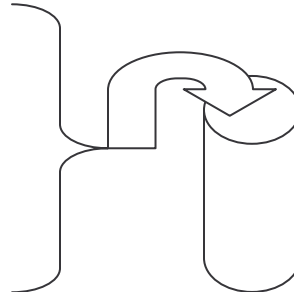
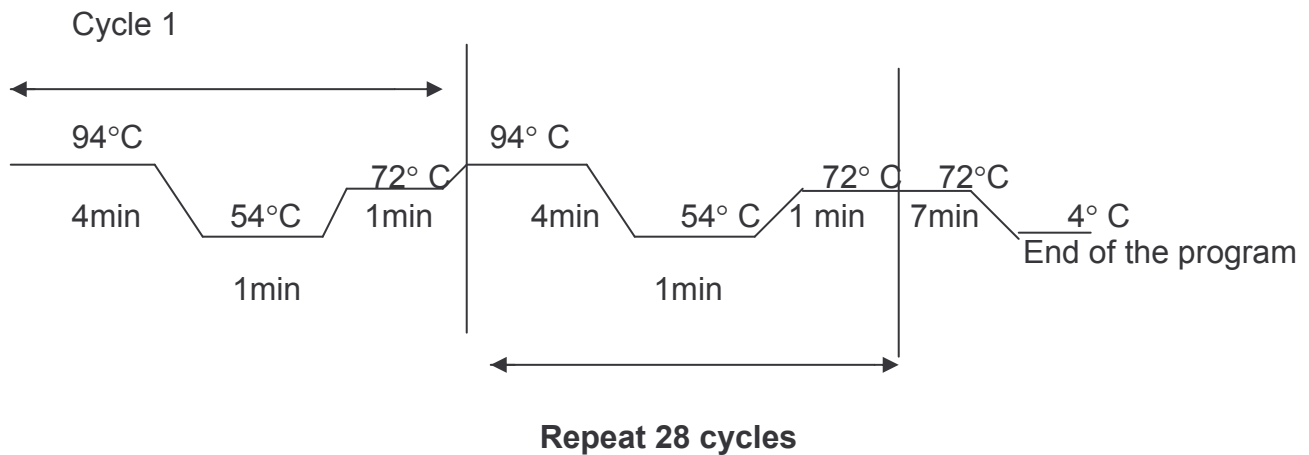


PCR amplification: the experiment consists two parts:

I. Generation the N-terminal:

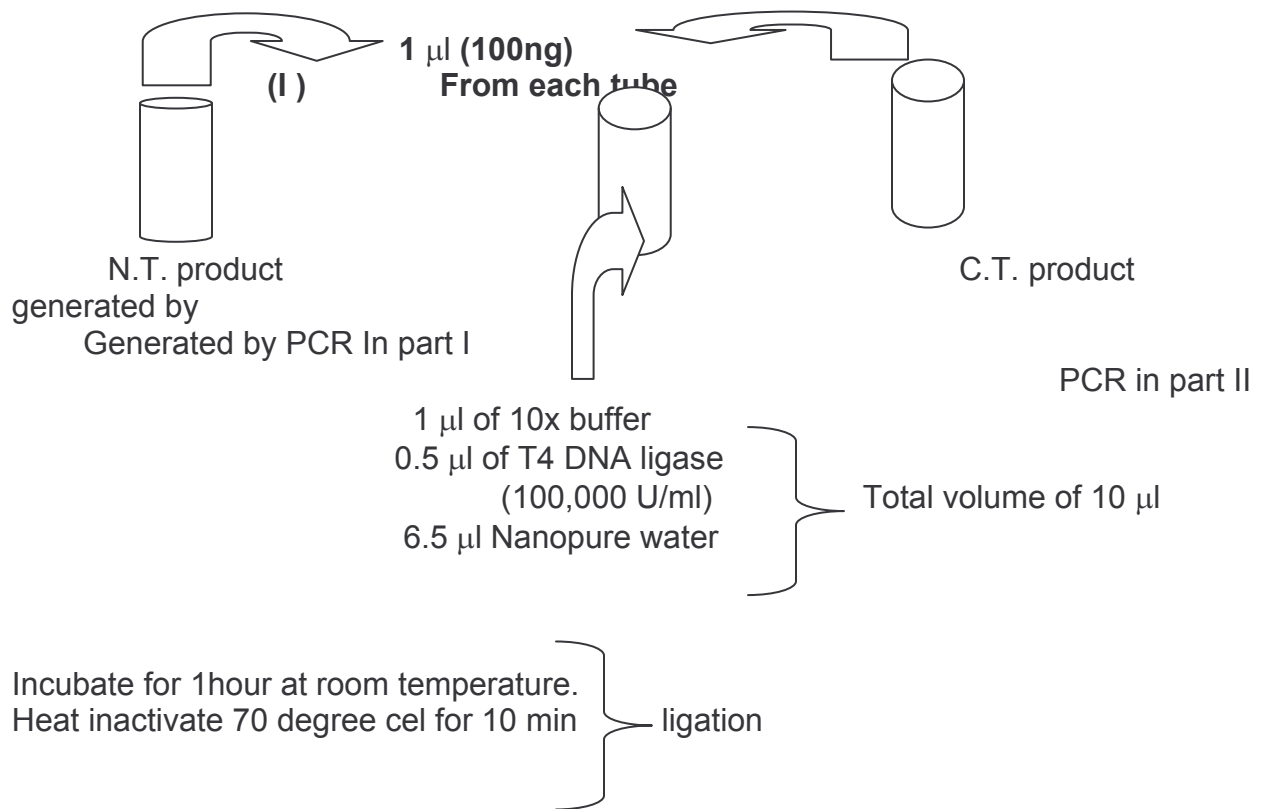
5ng template DNA
 2.5 µl forward primer (0.05microg/microliter)(10X stock)
 2.5 µl reverse primer (0.05microg/microliter) (10X stock)
 2.5 µl 2mM mgSO₄
 2.5 µl of 10X pfU buffer (stratagene)
 0.5 µl PfU DNA polymerase (5U/ µl)



Part(I)**II. Generation the C-terminal.****5ng template DNA****2.5 μ l forward primer (0.05 μ g/ μ l)(10X stock)****2.5 μ l reverse primer (0.05 μ g / μ l)(10Xstock)****2.5 μ l 2mM mgSO₄****2.5 μ l of 10X pfU buffer (stratagene)****0.5 μ l PfU DNA polymerase (5U/ μ l)****part (II)****Program:** Thermocycling parameters used

III. DNA purification after amplification

IV. ligation:



IV. Amplification of the ligated PCR product.

- 1 µl (10ng) of the ligated product
- 2.5 µl forward primer # I
- 2.5 µl reverse primer # IV
- 2.5 µl d NTP
- 0.5 µl 1100mM MgSO₄
- 2.5 µl 10X pfU buffer
- 0.5 µl pfu DNA polymerase

Use the same parameters of the above program to amplify

- V. DNA purification by gel clean-up
- VI. Restrict digest by the appropriate restriction enzymes (XmaI and EcoRI)
- VII. Clone in Yeast plasmid digested also by XmaI and EcoRI (treated with phosphatase before ligation)
- VIII. Transform in E.coli cells

- IX. DNA purification (miniprep)
- X. Sequence to check the frame
- XI. Yeast two-hybrid protocol to check for LZ-PNT P2 protein-protein interaction.